

UNIVERSITY OF GHANA

COLLEGE OF BASIC & APPLIED SCIENCES

**MOLECULAR CHARACTERIZATION OF ROTAVIRUSES FROM A SEMI-
URBAN COMMUNITY IN THE CENTRAL REGION OF GHANA**

BY

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**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN
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DECLARATION

I DELADEM KOFI AMEKUDZI, do hereby declare that with the exception of references to other research studies, which were duly acknowledged, this thesis is the outcome of my own research conducted at the Department of Biochemistry, Cell & Molecular Biology, University of Ghana, College of Basic and Applied Science under the supervision of Dr. Osbourne Quaye. Neither all nor parts of this project have been presented for another degree elsewhere.

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DEDICATION

To God be the Glory. Great things he has done.

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I am very grateful to the Almighty God for his wisdom, guidance, and protection throughout my life. I want to thank my supervisor, Dr. Osbourne Quaye for his immense guidance and patience throughout the project.

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ABSTRACT

Acute gastroenteritis (AGE) is an inflammation of the intestinal mucosa and is characterised by the onset of diarrhea with or without vomiting, fever, and abdominal pain. Rotaviruses are the leading cause of AGE in infants and young children resulting in severe dehydration, which can eventually lead to hospitalisation and death. In Ghana, diarrhea is implicated in the death of about 11% of all children under the age of 5 years, with rotavirus being the most responsible causative agent as with the case of most countries in the world. Currently, in Ghana, the prevalence of rotavirus is 28%. Rotavirus vaccine, Rotarix has been introduced into the immunisation system in Ghana to reduce morbidity and mortality due to the virus. This study was therefore designed to determine the prevalence of rotaviruses in a semi-urban setting, post vaccine introduction era.

Stool samples collected from children under five years of age with AGE, presenting at various Health Centres in the Breman Asikuma area in the Central Region of Ghana were used. The samples were tested for Group A rotaviruses using the Prowflow™ rotavirus enzyme Immunoassay (EIA) kit. Viral RNA was extracted from all samples using the Phenol-chloroform extraction method and electrophoresed on polyacrylamide gel for group characterization as well as the determination of RNA integrity. Negative EIA stool samples were then subjected to NSP3 qRT-PCR. Rotavirus genotypes of positive samples were determined by semi-nested RT-PCR with both gene and genotype specific primers, and the genotypes were further confirmed by Sanger sequencing. Further characterization of the virus was done by whole genome sequencing on the Illumina platform. Results showed that out of thirty-six (36) samples collected, three (9.0%; 3/36) tested positive by EIA. Electrophoretic analysis of the samples, also showed that two (2) of the samples were of the long electrophoretype and one (1) sample is of the short electrophoretype. Circulating strains were

found to be G9P[8] and G3P[6]. Full genome characterization of the selected strains gave Wa-like constellations.

The results of the study suggest that AGE due to rotavirus is relatively lower compared to the national incidence reported in literature.

CHAPTER ONE

1.0 INTRODUCTION

Diarrheal diseases are the fourth most common cause of death in children under 5 years globally (UNICEF, 2016). There has been a substantial drop in parasitic and bacterial diarrhea-related morbidity and mortality in the last decade due to improvement in sanitation, water supply and hygiene (Lundgren & Svensson, 2001). These improvements, however, have no impact on diarrhea of viral origin (Lundgren & Svensson, 2001).

Rotavirus has been recognised as the single most important cause of severe diarrhea in children less than five years old worldwide and an imperative general medical issue, especially, in developing nations (Liu et al., 2012; Thomas et al., 2017). Rotavirus infection is associated with vomiting, fever and diarrhea (Carlson et al., 1978; Parashar et al., 2006b). It imposes a devastating impact on children, infecting a large group of the population of children in Asia and Africa; reports attribute 215,000 mortalities in children less than 5 years of age to rotavirus yearly; with 107,500 of deaths happening in sub-Saharan Africa, (Tate et al., 2016). Until the introduction of the vaccine, rotaviruses accounted for 23% of all AGE related deaths in Ghanaian children under five years of age (Enweronu-Laryea et al., 2012).

Children, especially between the ages of 6 months to 2 years, are highly vulnerable to the disease (Glass & Parashar, 2014). Rotavirus transmission is via the fecal-oral route (Cortese et al., 2009). The virus can also be transmitted through contaminated food, water, and surfaces (Butz et al., 1993). Rotavirus has an incubation period of 1 – 3 days after which clinical features are presented. These clinical features are however not peculiar to rotavirus disease but share similarities with all gastroenteric related diseases. Globally, rotavirus infection is seasonal, peaking during the cool dry months (Mwenda et al., 2010; Patel et al., 2013). The epidemic

peaks in December in the USA and in Spain in January. The reason for this seasonality is currently uncertain.

Enzyme linked Immunosorbent Assay (ELISA or EIA), directed against VP6 protein is the recommended form of diagnosis replacing electron microscopy, which used to be the Gold standard. Other molecular techniques including Polyacrylamide gel electrophoresis (PAGE) and Reverse–Transcriptase (RT-PCR) which are used for further detection and characterization of the virus and have been proven to be equally sensitive (Zeng et al., 2008).

Treatment of rotavirus gastroenteritis is primarily aimed at replacement of lost fluid and electrolyte as it is only a supportive measure. Intravenous fluids are encouraged in severe diarrhea and nutritional therapy is also important in the reduction of mortality and morbidity. There are no antiviral agents for rotavirus treatment thus the public health strategy is vaccination which is preventive. Rotavirus vaccine development began in a bid to reduce and prevent global morbidity and mortality. An oral vaccine was thought to be the best choice for vaccine as it could mimic the natural route of infectivity of the wild rotavirus. Presently, there are two rotavirus vaccines; RotaTeq (Merck Vaccines, Whitehouse Station, New Jersey, USA) and Rotarix (GlaxoSmithKline Biologicals, Rixensart Belgium) that have been licensed and included in national vaccination programs in most developed and developing countries as an approach for the control of rotavirus associated mortality (WHO, 2009).

RotaTeq vaccine was licensed in the United States in the year 2006. It is a live oral vaccine, which is constituted of five reassortant (G1 – G4; P1A[8]) rotavirus strains developed from human and bovine parent rotavirus strains (Dennehy, 2008).

Rotarix, RIX4414 is a live attenuated human rotavirus vaccine. It employed P1A [8] G1 strain as the parent strain. It has an overall efficacy of 85% mainly against severe rotavirus disease (Kirkwood et al., 2017). It was first licensed in 2004 in Mexico and currently the vaccine of

choice for use in Ghana (Wang et al., 2015). Indian neonatal strain vaccine and G3 (RV3) neonatal strain vaccines are under developmental stages (Kirkwood et al., 2017).

Rotavirus studies have been conducted in the Northern and Southern regions of Ghana during the pre and post vaccine era (Armah et al., 2001; Binka et al., 2011; Binka et al., 2003; Damanka et al., 2016). However, no known rotavirus studies have been conducted in the Central region of Ghana and for that matter Breman Asikuma. Thus, this study was therefore designed to determine the prevalence of rotaviruses in the post vaccine introduction era in a semi-urban setting.

1.1 Problem statement

Rotavirus strain identification is an essential constituent of epidemiological surveys, disease distribution, vaccine efficacy monitoring and evaluation programs (Mukhopadhyaya et al., 2017). Current rotavirus studies have emphasized the need for continuous surveillance to provide adequate information on the circulating rotavirus strains, reassortment and recombination of strains in the population (Dennis et al., 2014; Doll et al., 2015; Wang et al., 2015). However, in Ghana several pre-vaccine and post-vaccine studies have been carried out. The majority of the studies were conducted in urban and rural areas. Most RV studies were done in the Greater Accra Region and the Northern Regions of Ghana (Armah et al., 2016; Armah et al., 2010; Armah et al., 2013; Armah et al., 1994; Armah et al., 2001; Binka et al., 2011; Damanka et al., 2016; Enweronu-Laryea et al., 2014a; Enweronu-Laryea et al., 2014b). However, there have been no reports on rotavirus studies in the Central region of Ghana and for that matter Breman Asikuma.

Breman Asikuma is a town in the Asikuma-Odoben-Brakwa district in the Central of Ghana. Breman Asikuma is the district capital. The district according to the 2010 Population and Housing Census has 112,706 (GSS, 2014) children between the ages of 0 – 4 years who form

15.4% of the total population of the district. The district is located between latitude 5° 51” and 5° 52” North and longitude 1°50” and 1°5” West (GSS, 2014). Breman Asikuma links Accra to Cape Coast and has a semi–urban settlement. In 2012, the rotavirus vaccine Rotarix™ was included in the national Expanded Program on Immunization to manage rotavirus-associated diarrhea. Thus, continuous surveillance across different parts of the country is pertinent.

1.2 Justification

A number of studies have revealed that the most effective means of lowering the rotavirus disease burden is by the inclusion of vaccines into the national immunization programs of countries. In Ghana, Rotarix™ vaccine was introduced into the EPI to manage diarrhea associated with rotaviruses in children under the age of 5 years. However, there is a lack of relevant data on the circulating strains in the post vaccine introduction era. Therefore, continuous surveillance is paramount to determine the impact of the Rotarix™ vaccine on the prevalence of rotavirus post vaccination in all regions of the country. Additionally, there is need to gather data on rotaviruses, which will help in a comparative analysis with circulating strains in other parts of the world.

1.3 Significance of the study

This study will provide relevant data on the strain diversity and genetic diversity because of reassortment and recombination of rotavirus strains. Most importantly, it will provide information on the circulating strains in the population and how related they are to other strains in the country and on the continent at large. Finally, recommendations from this study will help public health officials to better manage the rotavirus disease burden in the study area and in the entire country.

1.4 Study Objective

1.4.1 Aim

To determine the prevalence of rotaviruses in the post vaccine introduction era in a semi-urban area in the Central Region of Ghana.

1.4.2 Specific Objectives

1. To detect rotaviruses that are circulating in the population
2. To characterize the rotaviruses that are circulating in the population
3. To determine the relatedness of the strains detected to previously reported strains in the world.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Structure of Rotavirus

Rotaviruses are wheel shaped viruses that got their name from their distinct and complex shape (McClain et al., 2010). The viral name was derived from the Latin word “rota” meaning wheel. When examined by negative stain under the electron microscope, they are seen as wheel-like particles with small spikes and a well-defined rim (Fig 2.1). They are approximately 76.5 nm in diameter, non-enveloped double-stranded RNA viruses, with a segmented genome housed in the core of a triple layered capsid (McClain et al., 2010) (Fig 2.2). All eleven gene segments code for an individual protein, with the exception of gene segment 11 that codes for two distinct non-structural proteins (Estes & Kapikian, 2007). Of these 12 proteins, six are structural proteins (VPs) and the other six, non-structural proteins (NSPs). Electrophoresis of the RNA genome also allows for the classification of the virus into a Long (L) or Short (S) electrophoretotypes based on the migration patterns of gene segments 10 and 11. The capsid of the viruses comprises three concentric icosahedral protein coats.

The outer most protein coat is made up of the viral protein 7 and viral protein 4. Both of which catalyze the manufacturing of neutralizing antibodies (Aoki et al., 2009; Dormitzer et al., 2002; Malik et al., 2008). These two proteins also provide the basis for a dual classification system defining the G-types (glycoprotein) and P-types (Protease sensitive proteins). The VP7 constitute most of the outer capsid layer forming a shell around the virion (Aoki et al., 2009). The VP4 proteins produce protease-activated spikes used for attachment by the virus (Fig 2.2).

Trypsin-like proteases in the intestines result in the splitting of the VP4 protein into 2 polypeptides VP8* and VP5* (Arias et al., 1996; Ruggeri & Greenberg, 1991). The VP8*

makes the crown of the VP4 spike, while the VP5* makes the head plus base of the protein (Settembre et al., 2011). VP8 and VP5 are made up of consecutive neutralizing epitopes and surface-exposed neutralizing epitopes (Kovacs-Nolan et al., 2003; Larralde et al., 1991).

The viral protein 6 (VP6) constitute the center layer of the virion. It is the most abundant, immunogenic, and conserved region of the rotavirus. It has also been shown to induce heterotypic cross-protective immunity via eliciting T cell (CD4+) responses and circulating IgA antibodies, which kill the virus via intracellular activity (Esquivel et al., 2000; Estes et al., 1987; Franco et al., 2006; Tang et al., 1997). Based on antibody specificities to VP6 the virus is classified into seven groups (A-G). Groups A, B and C infect humans whereas groups D, E, F and G exclusively infect animals.

The viral protein 2 (VP2) forms the inner most layer and core capsid of the virion (McClain et al., 2010). Joined to its' interior is the viral RNA-dependent RNA polymerase and a capping enzyme (Lu et al., 2008). The non-structural proteins; NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6 are involved in various stages of genome replication, morphogenesis, stimulation of viral gene expression and the regulation of host innate immune responses (Estes & Kapikian, 2007).

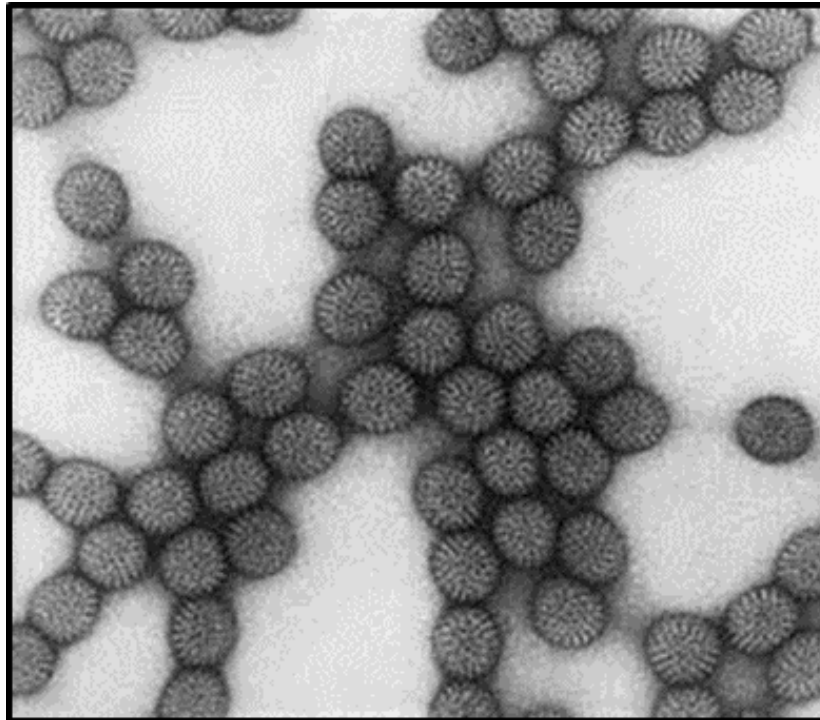


Figure 2.1: Transmission electron micrograph of intact rotavirus particles, double-shelled with well-defined rims Source: CDC/Dr. Erskine Palmer, 1978

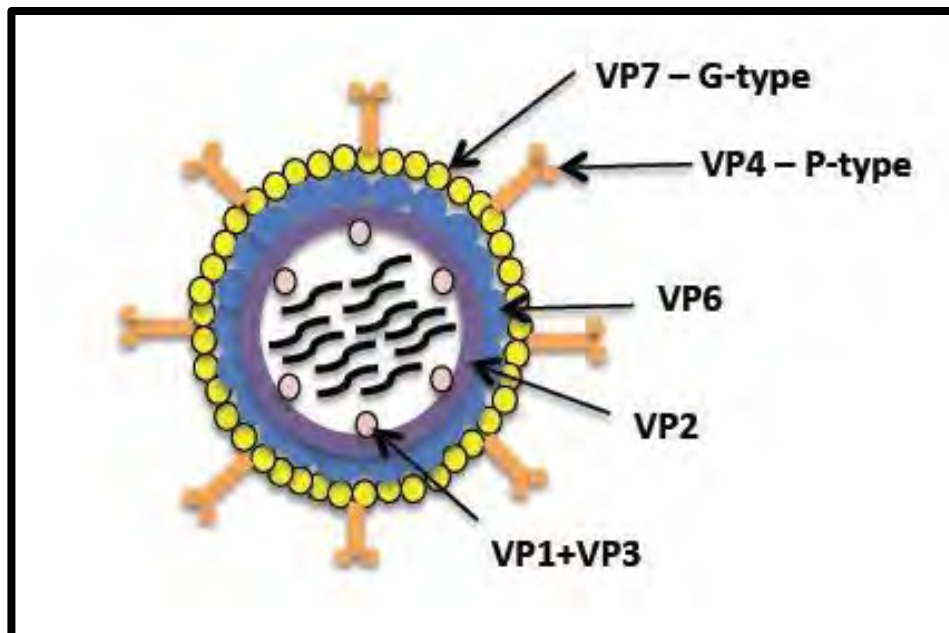


Figure 2.2: Schematic representation of the rotavirus virion. Adapted from (Aoki et al., 2009)

The rotavirus genome is approximately 18,500 bp in size and consists of eleven segmented dsRNA that is housed in the viral core capsid. All the genes, with the exception of the eleventh

gene of the virus are monocistronic encoding for six (6) structural (VP1-VP7) and six (6) (NSP1-NSP6) non-structural viral proteins.

2.2.1 Rotavirus Structural Proteins

The viral proteins are named conforming to their molecular weights, with the biggest being VP1 – 125 kDa and VP8 (proteolytic fragments of VP4) being the least at 28 kDa. The six (6) structural proteins form the multilayered capsid of the matured viral particle whilst the non-structural proteins are synthesized in the infected cell and perform roles in viral replication, pathogenesis or immune response to infection.

Viral Protein One (VP1)

It is the most conserved between rotaviruses of groups A and C (Bremont et al., 1992). The VP1 is encoded for by the gene segment 1 and is one of the 3 proteins (VP1, VP2 and VP3) that forms the core of the rotavirus particle. It has been suggested to function as an RNA dependent RNA polymerase of the virus. It has a size of 3302 bp and usually found as a complex with VP3.

Viral Protein Two (VP2)

It is encoded by the genome segment 2 and has been shown to be essential for the insertion of the polymerase enzyme VP1 and the guanylyl transferase (VP3) into the core of the virion (Vende et al., 2003). The VP2 forms an icosahedral shell that encapsulates the ds RNA of the virus, (Cohen et al., 1989).

Viral Protein Three (VP3)

The VP3 encoded for by gene segment 3 is a multifunctional enzyme, involved in mRNA capping by catalyzing the formation of the 5' cap structure on the viral plus-strand transcripts (Chen et al., 1999). This cap stabilizes viral mRNAs by protecting them from nucleases

Viral Protein Four (VP4)

The VP4 proteins protrude as spikes on the surface of the viral particle and is the resulting protein product of gene segment 4 (Gardet et al., 2006). VP4 is known to play a role in cell attachment and membrane penetration. As part of the outer capsid it is non-glycosylated but a haemagglutinin. In the enhancement of viral infectivity, this protein, in the presence of host's trypsin-like proteases is cleaved into two components VP5* (approximately 60,000 Daltons) and VP8* (approximately 28,000 Daltons). The fragment VP5* is a membrane penetration protein whereas VP8* fragment becomes viral haemagglutinin. VP8* has been suggested to bind to sialic acid receptors in the host cell membranes (Dormitzer et al., 2001).

Viral Protein Six (VP6)

The VP6 which is copious among all the viral proteins forms the middle layer of the rotavirus capsid structure and is encoded for by gene segment 6. This protein is important for virion assembly and has been shown to interact with VP2, VP4 and VP7 (Affranchino & Gonzalez, 1997; Crawford et al., 1994). The VP6 is the group determinant antigen of the rotavirus and is the protein currently used in laboratory diagnosis for rotavirus A infections.

Viral Protein Seven (VP7)

The viral protein 7 is the second most abundant capsid protein encoded by genome segments 7, 8 and 9 based on the strain (simian rotaviruses-genome segment 9; bovine rotaviruses-

genome segment 7; rhesus rotaviruses-genome segment 8) of infecting rotavirus (Estes et al., 1989). VP7, a glycoprotein forms the major outer capsid protein of the virion and is highly immunogenic inducing neutralizing antibodies (Ludert et al., 2002). It is used along with VP4 in the dual classification of rotaviruses.

2.2.2 Rotavirus Non-Structural Proteins (NSPs)

Non-Structural Protein One (NSP1)

NSP1, the product of genome segment five has been implicated in host range restriction in countering anti-viral response and in the suppression of apoptosis induction in the early stages of infection (Arnold & Patton, 2011).

Non-Structural Protein Two (NSP2)

NSP2 is a multifunctional enzyme, important for the development of the viroplasm and also essential in genome replication NSP2, in addition, interacts with NSP5 in viroplasm formation, VP1, and VP2 (Patton et al., 2006). This protein is coded for by gene segment 8.

Non-Structural Protein Three (NSP3)

NSP3 a 36.4 kDa protein, encoded by the 7th segment of the rotavirus genome. It is proposed to be associated with the translation of viral mRNA transcripts as well as the suppression of host protein synthesis (Poncet et al., 1993).

Non-Structural Protein Four (NSP4)

It is the enterotoxin associated with rotavirus induced diarrhea and is a protein encoded by segment 10. It is a 175 amino acid (aa) multifunction protein. It has been demonstrated to be essential in rotavirus replication, transcription and morphogenesis (Silvestri et al., 2005).

Non-Structural Protein Five and Six (NSP5 and NSP6).

Both non-structural proteins (NSP5 & NSP6) are encoded by genome segment 11. Published work by Afrikanova et al. (1996), indicates that NSP5 exists in multiple isoforms of 28 kDa to 32 kDa in a hyperphosphorylated form. It is firmly established that it interacts with NSP2 as a binding partner in the formation of viroplasms. It has also been shown to interact with VP1, VP2, and NSP6, (Hu et al., 2012). The particular role of NSP6 in replication has still not been ascertained though it has been proposed to be localize in the viroplasms (Rainsford & McCrae, 2007).

2.3 Rotavirus Pathogenesis

Post consumption, enterocytes which are cells situated at the end of the small intestine become infected with rotaviruses where they replicate within the cytoplasm (Fig 2.3) (Parashar et al., 1998). The triple-coated virus particles are transcriptionally non functional and are joined to the cellular membrane via VP8* (at the tip of the VP4 spike) after trypsin cleavage of VP4

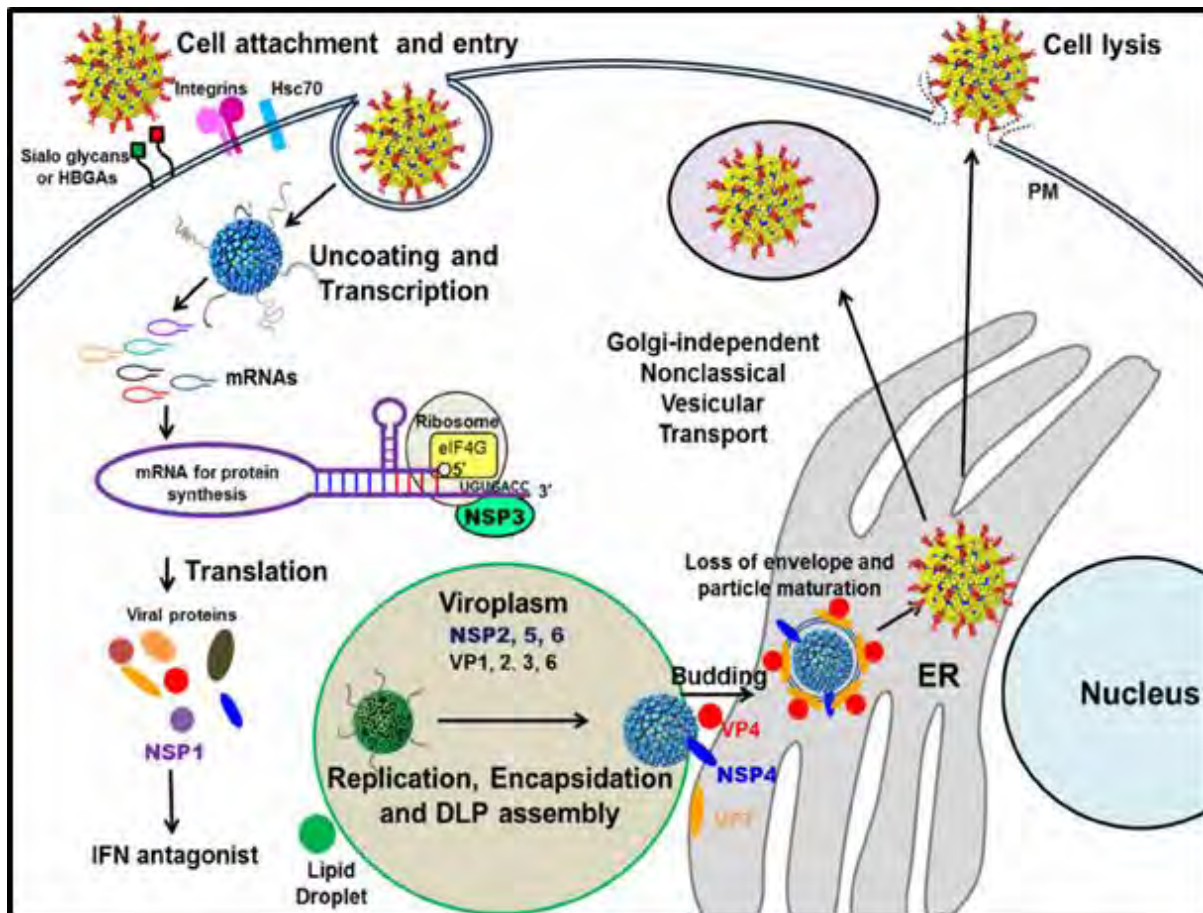


Figure 2.3: Rotavirus replication cycle (Hu et al., 2012).

into VP8* and VP5*. The triple-coated virus particle is conveyed to an early endosome by endocytosis, where the calcium levels results in uncoating of the virion from VP7 proteins and induce membrane infiltration by VP5*, ensuing in a transcriptionally functional dual-layered rotavirus particle (DLP) in the cytosol (Chemello et al., 2002; Ludert et al., 1986; Wolf et al., 2011). Directly proceeding the elimination of the external capsid and discharge to the cytosol, VP1 and VP3, polymerase complexes situated on the inferior side of the deepest coat, begin the transcription of messenger RNAs from all eleven genome segments (Lu et al., 2008; Mansell & Patton, 1990; McDonald & Patton, 2008, 2011; Patton et al., 1997; Patton et al., 1996).

At the foot of type I channel, transcription of RNA from double layered rotavirus particles occurs. It is placed in the five-fold apexes of the cover viral protein 6. The negative strands of

the double stranded RNA genome fragments are employed as templates for the positive RNAs during replication of the genome (Lawton et al., 2001). This is followed by further extrusion of capped messenger RNAs from the double layered particles by Type I conduits in the five-overlay corners and translation in huge inclusions made from NSP2 and NSP5 in the viroplasm. (+) RNA linked with viroplasm are packaged further into VP2 cores, this leads to the activation of viral protein one in other to form the nascent core (Silvestri et al., 2004). Concurrently NSP5 & NSP2 relate and control the gathering of diverse proteins to regulate the rally of the structural proteins (Chnaiderman et al., 2002; Jayaram et al., 2002; Jiang et al., 2006; Kattoura et al., 1994; Vende et al., 2003). In order to avoid, a premature development the VP7 proteins are an addition, the viral protein seven proteins are assembled precisely in the endoplasmic reticulum (ER) (Stirzaker & Both, 1989).

Prior to the formation of DLPs, VP6 molecules are made after VP6 molecules affix to the budding core (Berois et al., 2003). Concerning the triple-coated structure, DLPs make complexes with VP4 and NSP4, in the ER (endoplasmic reticulum), these further infiltrate the membrane of the ER (Gonzalez et al., 2000). TLP is created in a process whereby the membrane of the ER is separated leading to the accumulation of VP6 (Trask & Dormitzer, 2006). Various ways are involved in the release of TLPs from cells that are infected with the virus. No less than lysis, emission from the apical cell surface, and by the lytic enzyme containing organelle (Jourdan et al., 1997; Musalem & Espejo, 1985).

2.4 Rotavirus Disease Mechanism-Rotavirus Induced Diarrhea

Rotavirus antigen has been reported in the blood of an infected child. This is as a result of the movement of the viral particles (RNA) from the villi to the other parts of the body (Blutt et al., 2003). High levels of RV antigen and RNA have been reported in infected children. Averagely over 60% in the case of the RNA and 52% in the case of the antigen (Blutt et al.,

2003; Chiappini et al., 2005; Fischer et al., 2005; Moon et al., 2012; Nakagomi & Nakagomi, 2005). The virus was found in the cerebrospinal fluid of a patient (Iturriza-Gomara et al., 2011). It has also been reported in major body organs like kidney, spleen, bladder, and heart (Li & Wang, 2003; Lynch et al., 2003; Morrison & Czinn, 2001; Nakano et al., 2011). Culturing of infectious RV particles, from human serum has failed; this could be due to inhibitors. Inhibitors found in the serum and sometimes viral particles are responsible for the failure in culturing the RV from blood (Beisner et al., 1998; Tzipori et al., 1980). High antigen levels was observed to be linked to the existence of serum virion. Immunofluorescence and modified isolation strategy were used to identify the RV particles in HT-29 cells (Blutt et al., 2007).

The most significant symptom of RV infection, diarrhea could be because of various mechanisms, it can be secretory or probably osmotic (Hyser et al., 2010). The destruction of epithelial cells and differences in the calcium ion concentration has been illustrated as the mechanism of rotavirus diarrhea. Intracellular calcium ion concentration goes up to promote rotavirus replication, NSP4 has been implicated in the process. Higher calcium ion concentration results in the inhibition of Sodium co-transporters, and this leads to the reduction of the absorptive ability of the epithelium cells in the intestine. Osmolality of the small intestine increases because of increasing “unabsorbed organic molecules” leading to what is known as “osmotic diarrhea”. Nonetheless, studies have reported that nonstructural protein four protein is accountable for the increase in calcium ion concentration in the cytoplasm (Hyser et al., 2012). An interruption of closed junctions in intestinal absorptive cells, whereas it induces secretion in crypt cells (Morris & Estes, 2001). Furthermore, NSP4 can induce the production of serotonin in a type of endocrine cells known as enterochromaffin leading to the activation of the nervous system (Hagbom et al., 2011; Lundgren et al., 2000). Kaila et al. (1995), have demonstrated that oral rehydration solution (ORS) when used in the treatment of children

infected with rotavirus results in a corresponding reduction in the severity and length of diarrhea. Isotonic or hypotonic oral rehydration solution boosts rehydration by co transporters, as water accompany the gradient (Vesikari et al., 1987). To add to that complements like *Lactobacillus* (usually strain GG) (Majamaa et al., 1995; Pant et al., 2007) and zinc have been demonstrated to be useful in the reduction of RV diarrhea. (Dalgic et al., 2011).

2.5 Whole Genome Sequencing

Next generation sequencing (NGS) otherwise referred to as high-throughput sequencing is the term used to define a number of current sequencing techniques which produces millions of sequence reads (Chiu & Miller, 2016). NGS is a useful tool for characterization of all the eleven gene segments of rotavirus. These technologies are known to be effective and cost saving for the user. Thus enhancing greatly molecular biology research studies. In recent times, there are a number of different commercially available NGS platforms, which include Illumina (Solexa), Roche 454 (Roche Diagnostic, Indianapolis, IN), Ion Torrent Personal genome machine (Life Technologies, Carlsbad, CA) and Illumina HiSeq and Illumina MiSeq (Illumina, San Diego, CA) sequencers. Illumina MiSeq sequencers have a read length between 35 to 150 bp. The DNA is usually fragmented and adaptors added when using MiSeq (Goodwin et al., 2016). However, in the use of NGS for rotaviruses, extracted double stranded RNA is used to prepare NGS libraries using kits and reagents specific for NGS technology (Esona & Gautam, 2015). Bioanalyzer (Agilent Technologies, Santa Clara, CA), is used to ascertain the value of the library. It is then followed with sequencing using instrument specific kits.

Data analysis is done by the reporter program integrated to produce FASTQ formatted sequence data. Contigs are amassed from the acquired sequence reads via the de novo assembly command or map to reference in the CLC Genomics Workbench (CLC bio, Boston, MA) (WHO, 2009). The assembled consensus sequences of each gene are used to query the available

RVA nucleotide database in the GenBank by means of nucleotide BLAST or by submitting the sequences to RotaC for genotype characterization (Esona & Gautam, 2015). Finally, the complete genome for each sample is built from nucleotide sequences of 11 genome segments by MEGA version software (Nyaga et al., 2015).

2.6 Rotavirus Classification

Rotaviruses comprises one genus of the family *Reoviridae*. This genus is divided into various groups premised on the amino acid sequences of their VP6 protein (Matthijnssens et al., 2012c). Up to this point, eight distinct groups, A-H have been identified (Matthijnssens et al., 2012a). Groups A, B, C, and H infect humans, with the group A viruses being the most pertinent etiological agents of acute gastroenteritis (AGE) especially in children less than five years of age (Matthijnssens et al., 2012b). Group B rotaviruses are mostly linked with adult diarrhea and members within the group C and H are associated with diarrhea in children 4 – 7 years of age (Caul et al., 1990). Groups D, E, F, and G rotaviruses are found infecting avian species (Matthijnssens et al., 2011; Trojnar et al., 2010).

Presence or unavailability of certain definite epitopes on the VP6 gene further differentiates the rotaviruses into subgroups (SG) and includes subgroup I, subgroup II, subgroup I/II, and non-subgroup I/II (Thongprachum et al., 2009).

The outer layer proteins (VP7 and VP4) further classifies rotaviruses in a traditional dual classification system into G-genotypes and P-genotypes respectively. This classification system was founded on reverse transcription polymerase chain reaction (RT-PCR), in which unique genotypes can be deciphered via gene length, and sequenced (Gentsch et al., 1992; Gouvea et al., 1990). Currently, the Rotavirus Classification Working Group has described 35 G-genotypes and 50 P-genotypes based on the nucleotide sequence variations in both the Viral Protein seven and Viral Protein four genes in humans and animals.

As a result, of the segmented nature of the virus, genome reassortment and recombination events have been shown to occur between rotavirus strains leading to the formation of reassortant or new strains (Matthijnssens et al., 2008). This makes the traditional dual classification system not sufficient to capture the diversity that exists within this virus. A recent comprehensive nucleotide sequence – based classification, has been established and it encompasses the use of all 11 gene segments of the virus. This new system accounts for the complete genome by assigning genotypes to each of the gene segment based on nucleotide identity cut-off percentages. Each of the 11 genes (VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5) are designated with a letter for each gene type and a number (x) for each genotype [Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx] (Matthijnssens et al., 2008).

2.7 Rotavirus Epidemiology

Parashar et al. (2006a), reported that prior to the inclusion of RV vaccines into health care systems, between the year 2000 and 2004, over 500,000 children died yearly globally as a result of RV infections (Parashar et al., 2006a). Most of these deaths associated with rotavirus were reported to take place in developing and underdeveloped countries in the world usually in Africa and Asia. In those parts of the world, health care infrastructure and services are poor. (Parashar & Glass, 2006). By the time a child turns 5 years, that child is usually infected with rotavirus at least once. Most children are infected prior to the age of two years (Glass et al., 1996; Velazquez et al., 1996).

Rotavirus has been shown to have a distinct seasonal distribution, mostly in areas with a temperate climate. The peak months are usually the winter or early in the spring (Koopmans & Van Asperen, 1999). In the tropics and subtropics, the pattern of rotavirus disease is not distinct like that of Europe. The peak months are usually in the dry and cool months. However

during the entire year, widespread rotavirus infections can be observed (Hashizume et al., 2008; Luchs et al., 2013).

Studies conducted to investigate the variations in circulating RV genotypes has been done in a number of continents including Africa (Rudd et al., 2015) specifically in countries that solely use Rotarix™ vaccine, like Ghana. Preceding the introduction of rotavirus vaccine in Ghana, studies conducted, on the circulating rotavirus strains in Ghana. This is essential in determining the potential influence of the strains on vaccine efficacies. One of such epidemiological studies was conducted by Asmah et al. (2001) on Ghanaian children less than 5 years with acute gastroenteritis in the rural Upper Eastern Region during 1998 to detect the circulating rotavirus strains. She detected the rotavirus genotypes G1, G2, G3, P[4], P[6] and P[8]. The predominant genotype was identified to be a G3 strain, followed by G2 and G1 among the VP7 genotypes.

Additionally, before the introduction of the Rotarix™ vaccine in Ghana studies identified eight G genotypes (G1, G2, G3, G4, G8, G9, G10, G12) and three P genotypes (P[4], P[6], P[8]) with G1 been the most prevalent genotype (50.9%) among the P genotypes as shown in Fig 2.4. Binka and colleagues in 2003, reported that, G2P[6], G3P[4] and G9P[8] were the predominant rotavirus strains in Ghana (Binka et al., 2003) and a research conducted by Enweronu-Laryea et al. (2013) has it that the most predominant G/P genotype combination was, however, the universally common strain G1P[8] (28%), followed by G3P[6] and G2P[6]. Based on their findings, the others may be novel strains which could contribute to the diversity of rotaviruses in Ghana. Mixed G and P genotypes were also detected with their respective proportions as G-mixed (7.3%) and P-mixed (24.2%) (Enweronu-Laryea et al., 2013). They further recommended constant surveillance of rotaviruses in the post-vaccine era in Ghana.

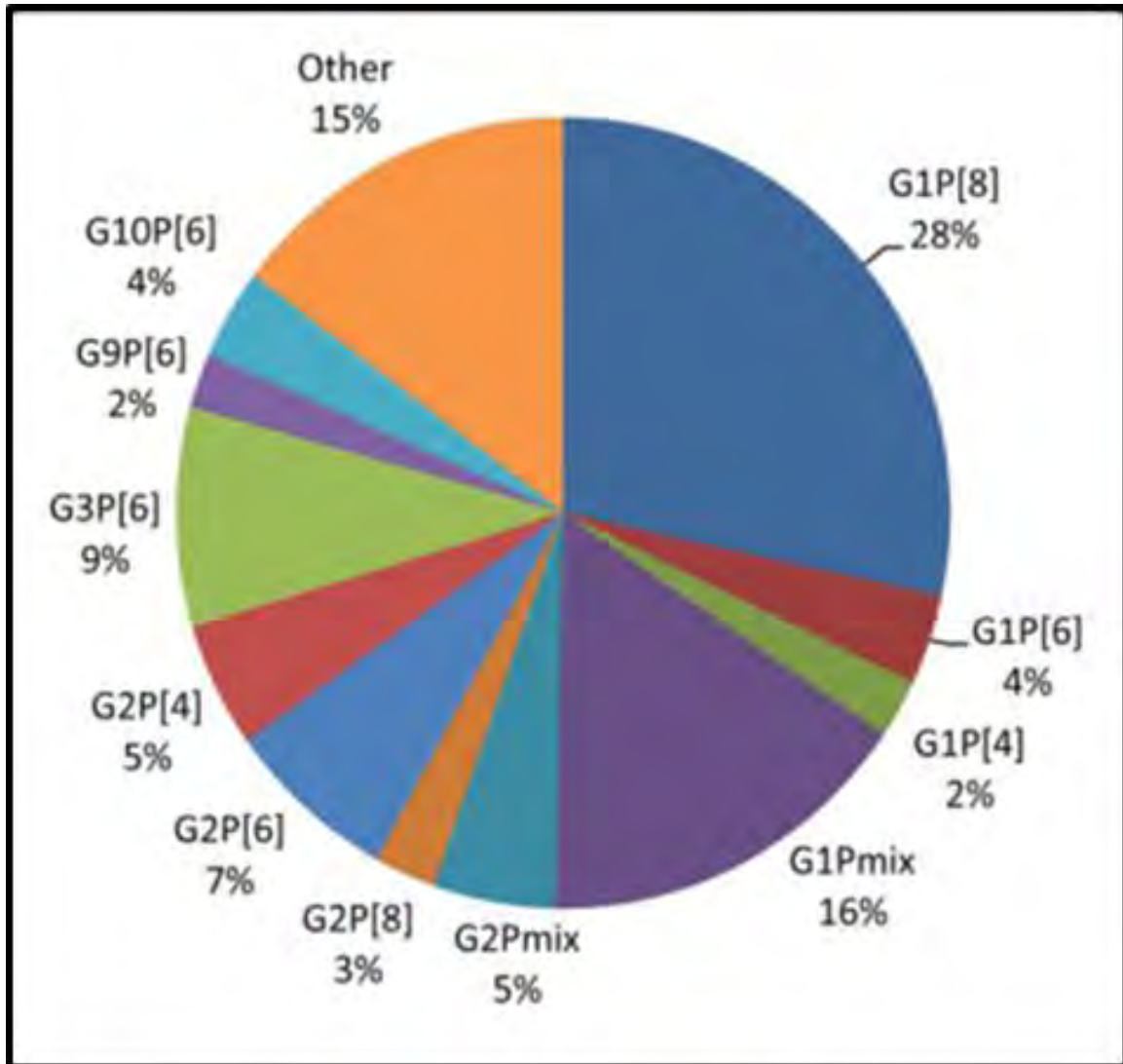


Figure 2.4: Prevalence (%) of rotavirus genotypes among 624 children hospitalized with AGE in Ghana: 2007 – 2011 (Enweronu-Laryea et al., 2013).

2.8 Rotavirus Vaccines

2.8.1 History

The advancement of RV vaccines from RV strains isolated from humans after research found that post-neonatal infections in infants that had experienced RV infection early in life (as newborns) were significantly milder and less frequent than in those infants experiencing their first RV infection. It was derived from neonates infected with RV. It was observed that post

neonatal RV infections reported in infants that have pre exposure to rotavirus infection were greatly less frequent and milder comparative to first timers of rotavirus infection (Bishop et al., 1983). The M37 vaccine was the initial vaccine of human origin. However, it was reported to stimulate a low protection level in infants even though it was found to be immunogenic (Flores et al., 1990; Midthun et al., 1991; Vesikari et al., 1991). A human RV was strain isolated from an asymptomatic child, that was shown to be shielded against harsh RV reinfections (Bishop et al., 1983). The RV3 vaccine produced was a low titer vaccine, unfortunately, not immunogenic (Barnes et al., 1997; Barnes et al., 2002). In August 1998, a live, oral tetravalent rhesus-human reassortant (G3P[3]) based rotavirus vaccine (RRV-TV, RotaShield®) was developed and licensed then used in routine vaccination of children in the United States. During the period of November 1998 to July 1999, a little over 600,000 children were vaccinated with the initial dose of RotaShield® vaccine (American Academy of Pediatrics Committee on Infectious, 2007). However, during a post vaccine surveillance study it was observed that RotaShield® vaccine was closely linked to intussusception in children. Therefore, it was recommended that the vaccine should be withdrawn from the market (Centers for Disease & Prevention, 1999; "From the CDC: Rotavirus vaccine withdrawn from market," 1999). Upon investigations, it was suggested that there exists a comparative risk of intussusception linked with increasing age of vaccination with the first dose of RRV-TV vaccine (Zanardi et al., 2001). Since then, 2 rotavirus vaccines Rotarix and RotaTeq have been successfully developed, licensed and recommended for global use ("Rotavirus vaccines WHO position paper: January 2013 - Recommendations," 2013).

Reassortant Human-Bovine Vaccine WC3 (RotaTeq)

Successive human-bovine reassortment was obtained and further advanced by Merck when initial WC3 vaccine showed low efficacy (Clark et al., 1988). On a bovine backbone, human

VP4 and VP7 were expressed by the reassortants. Various genotypes and their groupings were tested prior to the last composition made up of five RV reassortants (“RV5”) was licensed (Clark et al., 1990; Clark et al., 1996). Firstly, the RV5 was tried with G1 and G2 and tetravalent (G1-G4 and G1-G3 with P[8]) groupings in double-blind, placebo controlled studies in the US (Clark et al., 2004; Clark et al., 2003). G1 and G2 offered 73-87% protection against all rotavirus gastroenteritis but was statistically insignificant in the prevalence of diarrhea, fever and vomiting in patients who were given the vaccine inside forty-two days comparative to the placebo group (Clark et al., 2003). The tetravalent vaccine composed of genotypes G1-G3 and P[8] had identical efficacy vaccine to the bivalent vaccine: seventy-four percent protection against all rotavirus gastroenteritis and hundred percent against harsh rotavirus gastroenteritis (Clark & Offit, 2004). The ratio of children with vomiting or diarrhea was significantly bigger within patients who took the vaccine compared to those who did not receive the vaccine. To add to that, 4.4% of immunized children shed vaccine strains in their stools three to five days after the initial dose. Five of the patients shed the original vaccine strain whereas two shed a recombinant RV (Clark et al., 2004).

Thereafter, the vaccine composition was tried with another composed of genotypes G1-G4, prior to the addition of the P1A[8] genotype. This was done to achieve a bigger coverage and with the anticipation of increasing efficacy against all rotavirus gastroenteritis (RVGE). This vaccine constituent generated a 68-69% level of safety against every rotavirus gastroenteritis and 88-100% safety level against terrible rotavirus gastroenteritis (Vesikari et al., 2006a). A higher fever prevalence was observed in non-immunized children, although their absolute number was lower (Clements-Mann et al., 2001). A ninety-seven percent serological response was reported and also fifteen percent of the children shed vaccine strains in their stool samples with seven days post immunization (Clements-Mann et al., 2001; Vesikari et al., 2006c).

The breakthrough obtained during the bivalent composition studies was the basis used to compose the RV5 vaccine. The RV5 vaccine was made up of five reassortant human bovine RVs, (G1 and G2, G1-G3 with P1A[8], G1-G4, G1-G4 with P1A[8] and P1A[8]) composed of the five combinations, which were compared in a study conducted in Finland at three dosage levels, and observed to all have efficacy against rotavirus enteritis. There was no significant disparity in vomiting, fever or diarrhea among the various vaccine groups (Vesikari et al., 2006a). In addition, the middle dose level was chosen for the pentavalent composition as the eventual components. P1A[8] was also reported to produce a greater efficacy. The pentavalent vaccine offered protection against rotavirus gastroenteritis in the second and third post vaccination seasons (Vesikari et al., 2006a).

Between the years 2001 to 2005, seventy thousand infants in eleven nations were recruited in a research study on a grand scale, known as Rotavirus Efficacy and Safety Trial (REST). In the REST study the majority of the infants were tracked for a full RV season post vaccination. The outcome of the study suggested that the vaccine shielded against terrible rotavirus gastroenteritis and rotavirus gastroenteritis of any degree (hundred percent and seventy-three percent, respectively). Fourteen days post initial dose, a reduction by 86% of hospital visits for rotavirus gastroenteritis was observed. The vaccine was administered three times (doses), beginning with infants six to twelve weeks old with no increment in the risk of intussusception linked to the vaccine (Vesikari et al., 2006d).

RIX4414 Vaccine (Rotarix)

A vaccine candidate strain 89-12 was identified in an infant (Bernstein et al., 1990). Similar 89-12 strains were reported to provide diverse cross reactive neutralizing antibodies. The candidate vaccine strain 89-12 was reported to produce outstanding shield against RV (Bernstein et al., 1991; Ward et al., 1990). In order to develop a vaccine, the 89-12 strain was

grown in culture for thirty-three passages, and still maintaining immunogenicity (Bernstein et al., 1999). Eighty-nine percent efficacy was observed against and gastrointestinal infection and a hundred percent efficacy against devastating forms of gastroenteritis but was observed to be mildly in children three to five months of age. Following the post licensure of the 89-12 strains by GlaxoSmithKline (GSK), a sole rotavirus was selected at the end of passage 33, accompanied by twelve passages in Vero cells, leading to the formation of functional vaccine known as Rotarix™ vaccine, named as strain RIX4414 (Ruiz-Palacios et al., 2006; Vesikari et al., 2004).

2.8.2 The uses of rotavirus vaccines

In the year 2006, RV vaccines were incorporated into the national vaccination programs of several nations. Some of the countries include Australia, Austria, USA, Belgium and Brazil. In Ghana, Rotarix™ was included in the national immunization program in 2012 (Abbott et al., 2012). Similarly, in Finland, RotaTeq® was incorporated into the national immunization schedule in the year 2009. Moreover, prior to the introduction of RotaTeq®, Rotarix™ was utilized, with twenty-two percent scope between 2006 and 2007 and 35% scope in 2007-2008 (twenty-nine percent Rotarix™ and six percent RotaTeq®) (Rasanen et al., 2011). As of January 2014, fifty-three nations around the globe have included RV vaccines in their national immunization programs and various nations like Germany, Thailand, Canada, and provincially in the UAE.

2.8.3 Impact of Rotavirus on burden of disease

The inclusion of rotavirus vaccines in the national immunization programs of countries was reported to be the characteristics period of rotavirus infection. A ten years surveillance study conducted by the US Centers for Disease Control and Prevention (CDC), showed a number of outcomes. The start of the rotavirus season was hindered by two to four months in the initial

year post mass immunization. In addition, the RV season was cut short by 12 weeks throughout the six previous years prior to when rotavirus vaccines became accessible. Moreover, the season was extended again in the proceeding (second post-national immunization program) year (Tate et al., 2013). Similarly, in epidemiological studies conducted in Belgium and Brazil, rotavirus start season was moved by one to two months post introduction of rotavirus vaccines (Curns et al., 2011; Hanquet et al., 2011; Safadi et al., 2010). This type of change has not been witnessed prior to the introduction of vaccines, it is possible that the introduction of rotavirus vaccines has influenced rotavirus seasonality (Turcios et al., 2006). In the tropics, in nations like Brazil, rotavirus gastroenteritis took place throughout the cool and dry seasons, but widespread rotavirus infections or epidemics may take place during the entire year (Hashizume et al., 2008; Luchs et al., 2013). A change in the start or reduction of rotavirus season has not been noticed in Brazil (Luchs et al., 2013).

2.8.4 Effectiveness of rotavirus on gastroenteritis cases

Many European countries utilize both rotavirus vaccines, Rotarix™ and RotaTeq® which have proven to drastically decrease the number of hospital visits and outpatient hospital admission for rotavirus gastroenteritis. In Belgium, a sixty-one percent decline in rotavirus gastroenteritis cases in the first year of vaccination (Hanquet et al., 2011) which resulted in a 80% reduction of rotavirus gastroenteritis cases among eligible infants (two to twenty-four months of age) (Raes et al., 2011). Likewise, in Austria, rotavirus gastroenteritis-related hospital visits dropped by 74% in children fit for vaccination (Paulke-Korinek et al., 2010). A research study conducted in Brazil, estimated Rotarix™ vaccine efficacy to be eighty-five percent, with a major drop in clinic visits and death rates as a result of gastroenteritis of any origin in infants less than 1 year of age (by forty-eight percent and fifty-four percent, respectively) (Linhares & Justino, 2014). In Australia, where both rotavirus vaccines are in use, rotavirus

gastroenteritis-related clinic visits were lowered by 68-93% in infants below 1 year of age (Buttery et al., 2011).

In the United States, post introduction of the RotaTeq® vaccine reported a drop of 74-85% in hospital admissions of children for rotavirus gastroenteritis cases. Furthermore, a research carried out in the US, RotaTeq® vaccine was observed to effectively lower rotavirus gastroenteritis related hospital visits after the first two immunizations (Wang et al., 2013). Hospital admissions because of rotavirus gastroenteritis were lowered by 88% after the initial dose and 94% after the second dose, while health-care visits as a result of gastroenteritis of any cause were lowered by forty-four percent after the first dose and forty percent after the second dose (Wang et al., 2013).

Indirect effect in unvaccinated children

The introduction of rotavirus vaccines has lowered rotavirus gastroenteritis cases in unvaccinated infants. In Belgium, in the second post-NIP year, the total of rotavirus gastroenteritis cases in children past eligibility to be vaccinated in the national immunization program was lowered by sixty-four percent. Australia which uses a combination of both rotavirus vaccines, was estimated to have hospital visits for rotavirus gastroenteritis lowered by over 50% in children above 2 years of age (not qualified for rotavirus vaccination in the national immunization program). In the US, the usage of, mainly RotaTeq® vaccine was shown to lower cases by 42.45% among kids too old or too young to be incorporated in the rotavirus national immunization program (Tate et al., 2008).

2.8.5 Shedding of true vaccine viruses and vaccine-derived reassortants

Many pre-vaccine licensure studies have evaluated the phenomenon of shedding of vaccine strains. Shedding of RotaTeq® viruses has been found to be a rare occurrence, while Rotarix™

is usually shed right after the initial dosage (Vesikari et al., 2004). Outcomes of these studies of RotaTeq® reported a 13% shedding in vaccinated infants after the initial dose, 0-7% and 0.4% after the second and third dose respectively (Vesikari et al., 2006b). Concerning both research findings, the shedding of rotaviruses was determined by employing the viral culture technique with plaque assay and electropherotyping. In addition, different investigations from the post-licensure period that use ELISA have illustrated the genuine shedding rates to be substantially higher. Work done by Yen, Jakob, et al. (2011), detailed that 21% of inoculated newborn children were identified to have vaccine strain in stools assembled amid the initial nine days after the primary vaccination (Yen et al., 2011b). In a similar report, the viral load was resolved to be between 4.5×10^7 and 7.0×10^{12} duplicates for each gram of stool (Yen et al., 2011a). Hsieh et al. (2014) investigated the shedding of both rotavirus vaccines after each immunization using the ELISA and RT-PCR techniques. Post-RotaTeq® vaccine immunization 94% and 56% of children (RT-PCR and ELISA, respectively) shed the virus at some stage throughout the period of the initial twenty-eight days after the initial immunization. Shedding was uncommon after the second dose (67% and 9.3%) and after the third dose (62% and 8.1%).

Hsieh et al. (2014), in same research, reported shedding rates after vaccination with Rotarix™ were comparable. After the initial vaccination, 43% and 94% of kids (ELISA and RT-PCR, individually) shed the rotavirus vaccine strains in their stools reducing to 25% and 53% after the second measurement (Hsieh et al., 2014). Pre-licensure contemplates, announced the shedding of the Rotarix™ virus as extraordinary, with 21-61% of inoculated kids shedding the vaccine strains in their stools amid the initial 7 days continuing the primary vaccination and 11-21% after the second measurements (Dennehy et al., 2005; Phua et al., 2005; Ruiz-Palacios et al., 2007).

Vaccine-originated viruses and new reassortants

Since the introduction of RotaTeq® vaccine, shedding of vaccine derived twofold reassortant rotavirus has been described from Australia and the US. In the year 2009, the principal vaccine derived human-bovine twofold reassortant rotavirus was discovered. An unvaccinated boy was detected to shed vaccine-originated rotavirus in his stools after a hospital visit for symptoms and signs of gastroenteritis (Payne et al., 2010). After full genome characterization of every one of the 11 rotavirus genome fragments, the rotavirus was found to be a novel vaccine determined reassortant G1P[8] (vdG1P[8]) rotavirus gotten from two unique vaccine strains G1P7[5] and G6P[8]. The beginning of contamination was recommended to be from his recently immunized sibling, who demonstrated no indications of gastroenteritis (Payne et al., 2010). Thereafter, a study conducted by Donato et al. (2012) on children hospitalized for acute gastroenteritis after vaccination with RotaTeq® vaccine (Donato et al., 2012). They discovered after the study that thirteen kids (21%) were shedding RotaTeq® vaccine rotaviruses in their stools, and in 4 of them, a novel vaccine derived twofold reassortant was discovered. The rotavirus was recognized via genotyping and sequencing of VP3, VP4, VP6, and VP7 genome segments. The nearness of vdG1P[8] was believed to be conceivably connected with changed immune capacity, as a portion of the infants had fundamental restorative conditions (Donato et al., 2012).

Curiously, the reassortant between P[8] VP4 and G1 VP7 proteins was discovered before in the pre-licensure investigations of the tetravalent vaccine. To add to the vaccine-vaccine reassortants originating from the RotaTeq® immunization, another wildtype reassortant has been reported in Nicaragua in two RotaTeq® inoculated newborn children. The new reassortant has a specific genome constellation for a wildtype G1P[8] virus, but the NSP2 gene segment is similar to the cognate gene segment in RotaTeq® vaccine (Bucardo et al., 2011).

Boom et al. (2012), studied RV VP7, VP4, and NSP2 gene segments and found them to be similar to Rotarix™ vaccine viruses discovered in stool from an unvaccinated six month-old child. The child was on admission at the hospital for symptoms such as acute gastroenteritis and no other pathogen associated with gastroenteritis was identified; moreover, the genesis of the Rotarix™-derived virus was not clarified (Boom et al., 2012). Similarly, in the US, Rotarix™ was surprising detected in an unvaccinated child with acute gastroenteritis in an area using exclusively RotaTeq® (Payne et al., 2013). Prior to that, the horizontal transmission of vaccine strain in twins has been investigated by Rivera et al. (2011), the outcome points to the fact that horizontal transmission is a usual phenomenon, taking place in fifteen of eighty twins. Interestingly, not one of these transmissions resulted in rotavirus gastroenteritis in the affected children (Rivera et al., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

All chemicals were standard chemicals and were obtained from commercial companies. Reagents for the polyacrylamide gel electrophoresis were obtained from (Bio-Rad Laboratories, USA and California). The RT-PCR kits were obtained from (Promega, USA & Fitchburg). Agarose and Tris base, TAE buffer for agarose gel electrophoresis were obtained from Inqaba Biotech, South Africa. Rotavirus Proflow™ kit used for sample screening was obtained from Pro-Lab Diagnostics, UK & Bromborough.

3.2.1 Ethical Approval

This research study was a molecular characterization of group A rotaviruses obtained from stool samples collected as part of an Master of Philosophy research work in rotavirus surveillance in the Central Region of Ghana. The Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon approved the study. Written informed consent for the collection of stool samples for testing of rotaviruses and characterization of detected rotavirus strains was obtained from the children's guardians/parents before sample collection.

3.2.2 Study sites and Sample Collection

Breman Asikuma is a town in the Central Region of Ghana. It is somewhere in between Accra and Cape Coast. Thus it links Accra and Cape Coast perfectly. It is a semi urban town. It falls on Longitude -0.994° and Latitude 5.583° . The main health center for this study was Our Lady of Grace Catholic Hospital Asikuma. The hospital is located at the center of Breman Asikuma

and has storage facilities for proper maintenance of cold chain transport. The samples were collected from September 2015 to August 2016.

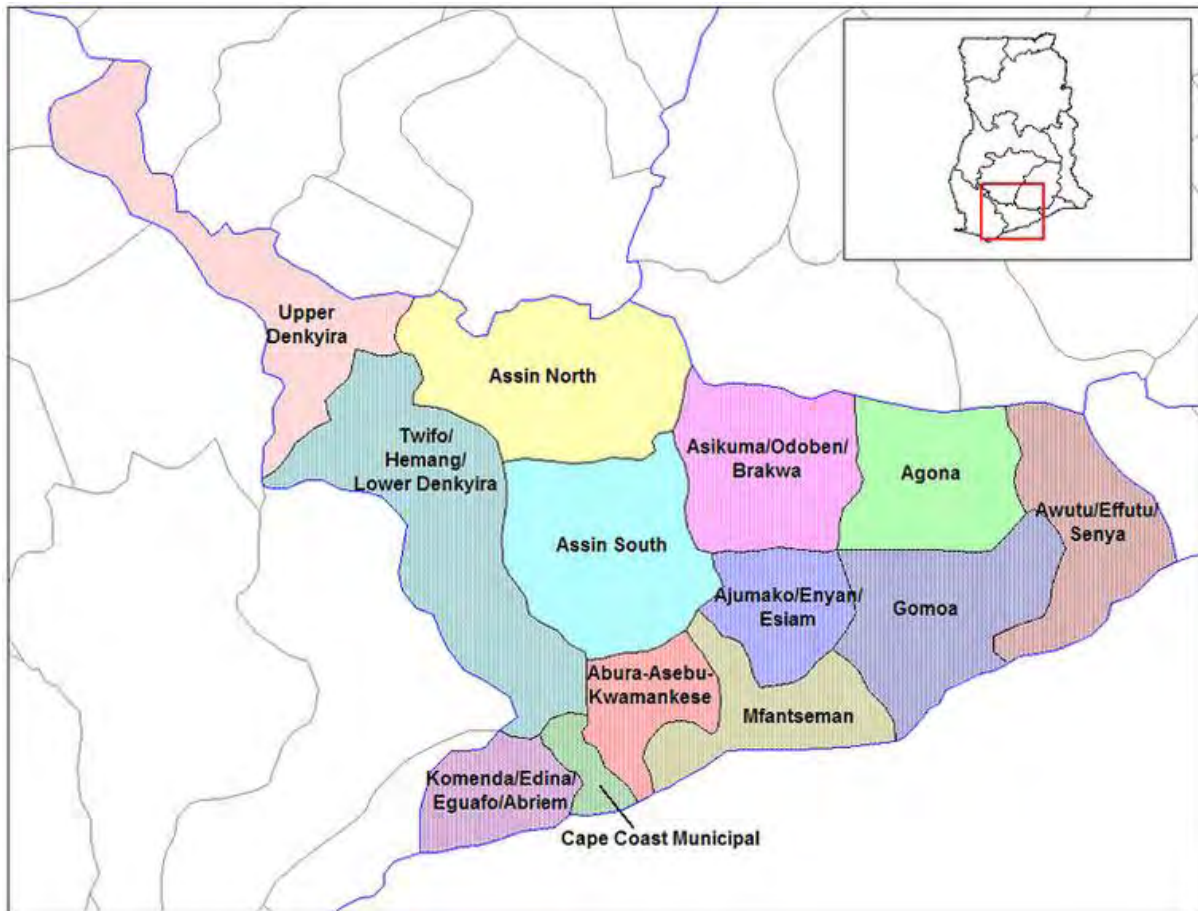


Figure 3.1: The map of Ghana and the Central region showing Breman Asikuma. Source (GSS, 2010)

Children under the age of 5 years presenting with diarrhea at the various Health Centers in the Breman Asikuma area were included in this study. A total of 36 stool samples were collected from these health centers. Samples collected were transported to the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, and stored at -20 °C until ready to be analyzed.

3.3 Sample Preparation and processing

Ten percent (10%) stool suspension was made by the addition of 1.0 g of stool sample to 900 µl of phosphate-buffered saline (PBS) of pH 7.2. The prepared suspensions were vortexed and centrifuged; then stored at 4 °C until the time of use.

3.4 Rotavirus Antigen Detection

The samples were screened using the Proflow™ Rotavirus test kit. The kit is a single rapid membrane immunoassay which is used in the qualitative detection of rotavirus antigens. The test works via capillary action and reaction with specific conjugates on the test strip. As an indication of positivity specific antibodies present on the membrane will capture the colored conjugate resulting in a specific color of the test line. The presence of a green line on the tests strip suggests the validity of the test, showing that the right volume of sample was added and that the reagents were functioning properly. All samples to be tested were allowed to equilibrate at room temperature before the test was carried out following the manufacturer's instructions.

A stick provided in the kit was introduced 2-3 times into the stock stool sample to pick up approximately 100 mg of the stool. The stick was put back into the sample preparation device with its buffer. The device was then shaken to ensure good sample dispersion in the buffer. The Proflow™ Rotavirus test strip was removed from its sealed pouch. The top of the sample preparation device was broken and 4 drops of the resulting mixture were dropped on the sample well portion of the test strip. The results were read after 10 min.

3.5 Viral RNA Extraction and Purification

A 1 M Sodium acetate (NaOAc) containing 1% sodium dodecyl sulphate (SDS), pH 5.0 was equilibrated in a water bath at 37 °C. Fifty microliters (50 µl) of the 1 M NaOAc containing 1% SDS was added to 500 µl of the 10% stool suspension, and was vortexed for 10 sec and

incubated for 15 min at 37 °C. Equal volumes (500 µl) of phenol/chloroform (1:1), was then added and vortexed for 1 minute and incubated at 56 °C for 15 min. The tubes were opened and immediately resealed prior to vortexing to release built in pressure in order to prevent the tube from popping open when vortexing. After incubation, the tubes were then vortexed for 1 min and centrifuged for 3 min at 12,000 revolution per minute (rpm). The supernatant was carefully removed containing the dsRNA and was placed in a clean 2.5 ml of microtube.

A volume of 500 µl of 6 M GITC was added to the recovered suspension and vortexed for 30 sec. It was then centrifuged at 12,000 rpm for 5 min. RNaid matrix was vortexed very well prior to use and 10 µl of the matrix was added to each sample and was incubated at room temperature on a rocker for 15 min.

Centrifugation was then carried out at 5,000 rpm for 20 sec and the supernatant was discarded. Four hundred microliters (400 µl) of RNaid wash buffer was added to the pellets and gently resuspended with a pipette. Centrifuged for 30 sec at 12,000 rpm and the supernatant was discarded.

The last step was repeated using 100 µl of RNaid wash buffer. Centrifugation was carried out at 12,000 rpm for 60 sec. The supernatant was discarded. Blotting was then carried out to get rid of excess ethanol. The pellets were then resuspended in 50 µl of Diethyl dicarbonate (DEPC) treated water. It was incubated at 65 °C in a water bath for 10 min to enable elution of RNA from beads. Centrifugation was then carried out 12,000 rpm for 3 min. The supernatant was carefully transferred into a sterile Eppendorf tube. It was then stored at -20 °C until needed.

3.6 Polyacrylamide Gel Electrophoresis (PAGE)

In order to confirm the presence of rotavirus RNA in the extracted product, a 10% polyacrylamide gel was prepared according to a protocol described by the ("Meeting of the

Strategic Advisory Group of Experts on immunization, October 2009 - conclusions and recommendations," 2009). Ten percent (10%) resolving acrylamide solution was prepared by mixing 9.9 ml of distilled water, 6.3 ml of 30% acrylamide, resolving buffer of pH 8.8, 10 μ l of TEMED and 282 μ l of 10% ammonium persulfate (APS).

The glass plates that make up the electrophoretic chamber were cleaned and subsequently wiped with absolute ethanol. The glass plates were then assembled according to the manufacturer's instructions in a gel caster. A 10% resolving acrylamide solution was prepared and pipetted in between the glass plates; it was overlaid with Isopropanol and was allowed to set for 45 min after which the isopropanol was poured off and the gel washed twice with distilled water.

A 4% stacking acrylamide solution was prepared by mixing 5.1 ml distilled water, 1.2 ml 30% acrylamide stock, 0.9 ml stacking buffer of pH 6.8, 4 μ l TEMED and 112 μ l of 10% APS. The stacking acrylamide solution was pipetted in between the glass plates on top of the resolving gel and to create loading wells a comb was positioned immediately and the gel allowed to set for another 45 min. The comb was carefully removed and the glass plates assembled in the electrophoretic chamber. Running buffer was added to the bottom reservoir of the electrophoretic tank and the wells were also filled with the buffer.

Fifteen microliters (15 μ l) of extracted RNA was mixed with 10 μ l of loading dye and loaded into the sample wells and the electrophoresis carried at 150 volts for 3 h.

3.6.1 Silver Staining

To view the electrophoretic patterns of the RNA electrophoresed the polyacrylamide gel was stained using the silver nitrate staining method described by WHO (2009). Fixing solution 1 was prepared by adding 80 ml of ethanol and 10 ml of acetic acid to 110 ml of distilled water.

Fixing solution 2 was prepared by adding 20 ml of ethanol and 1 ml of acetic acid to 180 ml of distilled water. Silver nitrate solution was prepared by dissolving 0.37 g of silver nitrate in 200 ml of distilled water. Stopping solution was prepared by adding 10 ml of acetic acid to 200 ml of distilled water. Developing solution was prepared by adding 2 ml of 36% formaldehyde to 250 ml distilled water and 7.5 g of sodium hydroxide was dissolved in the resulting solution just before it was used.

The running buffer was poured out of the electrophoresis chamber; and the gel carefully removed from the glass plates and placed in distilled water. The water was aspirated and fixing solution 1 was poured onto the gel and rotated at room temperature for 30 min on an orbital shaker. Fixing solution 1 was aspirated and fixing solution 2 added to the gel and rotated for 30 min on an orbital shaker at room temperature. The fixing solution 2 was aspirated and the silver nitrate solution added and rotated for 30 min at room temperature on an orbital shaker. The silver nitrate solution was aspirated and the gel washed with distilled water twice for 2 min each. Approximately 50 ml developing solution was added to the gel and was agitated by hand for 30 sec to remove black precipitates that were present. The silver nitrate was aspirated and the remaining was added and agitated by hand until the RNA bands were visible.

The developing solution was drained off and stopping solution added to prevent further color development. This was rotated for 10 min at room temperature.

3. 7 Synthesis of Complementary DNA (cDNA) and Amplification of VP7 and VP4

Genes

Purified dsRNA, VP7 and VP4 gene specific primers (working concentration), AMV Reverse-transcriptase and buffer, and 10 mM dNTPs were retrieved from storage and kept on ice. The 0.2 ml thin walled PCR tubes were labeled for each amplification reaction, indicating sample number, specific reaction type, and date. The negative control was also included. The sample

details were then transferred to RT-PCR worksheet. The RT master mix volumes were then calculated for the number of reactions and RT master mix prepared in a 1.5 ml labeled microtube on an ice bath and gently mixed.

One microliter (1 μ l) each of sense and antisense gene-specific primers was added to 8 μ l of dsRNA template in PCR reaction tubes and span. A negative control (double-distilled water) was included. Denaturing was done in a boiling water bath for 5 min and all reaction tubes were transferred immediately to an ice bath for a maximum of 5 min. A volume of 3.2 μ l of RT master mix was added to each tube and span. In a reverse transcription step, the tubes were then incubated at 42 °C for 20 min. GoTaq DNA Polymerase (Promega, U.S.A., California) and its buffer were retrieved from storage and kept on ice. The volumes for the polymerase chain reaction master mix (PCR-MM) required were calculated and the details recorded on the RT-PCR worksheet. A PCR-MM was then prepared in a labelled 1.5 ml Eppendorf tube and kept on ice. 36.8 μ l of the prepared PCR-MM was added to each reaction tube and flushed down. The tubes were then placed in a thermocycler and run at the following conditions; an initial denaturation at 94 °C for 2 min, followed by 30 cycles (denaturation at 94 °C for 1 min, annealing at 42 °C for 2 min, and extension at 72 °C for 3 min). A final extension at 72 °C for 5 min. The PCR products were electrophoresed alongside a 100 bp DNA ladder on a 1.5% agarose gel containing 0.5 μ g/ml TAE buffer pH 7.9;120 V for 1 h. The results obtained were documented via photography.

3.7.1 Genotyping of Amplified VP7 and VP4 genes

GoTaq DNA Polymerase, PCR buffer, VP7 and VP4 genotype specific primers and 10 mM dNTPs were retrieved from storage and kept on ice. PCR tubes were labelled indicating sample number, specific reaction type and date of reaction. Two microliters (2 μ l) of cDNA was added to the labeled tubes. The volumes for the PCR-MM was then calculated for the number of

reactions. Genotyping PCR-MM was then prepared in an appropriately labelled Eppendorf tube on ice. The required volume of PCR-MM was added to the tubes to make up a total reaction volume of 50 μ l. The tubes were then placed in the thermocycler and the previous reaction conditions were employed. The PCR products were electrophoresed alongside a 100 bp DNA ladder on a 1.5% agarose gel containing 0.5 μ g/ml TAE buffer pH 7.9;120 V for 1 h. The results obtained were documented via photography.

3.8 Sanger Sequencing

3.8.1 Cycle sequencing using ABI 7500 Fast DX Instrument

Twenty micro molar (20 μ M) of the primers were diluted to 2 μ M with DEPC treated water for cycle sequencing. A primer mix was made for 150 reactions by adding 300 μ l nuclease free water, 300 μ l of Big Dye (V.3) (Thermofisher Scientific, U.S.A. & Waltham) and 300 μ l of 5X buffer to a master tube to give a total volume of 1200 μ l. After that, 2 μ l of purified cDNA was placed in a 1.5 ml PCR tube and was labeled according to the template sheet of the plate after which 2 μ l of the 2 μ M primer was added to the templates. After the addition of 2 μ M to the templates, 6 μ l of the master mix was then added to make a total volume of 10 μ l. The samples in the tubes were span for few seconds to flush down. The samples were then placed in the the thermocycler and were run. The reaction was carried out with an initial denaturation at 96 °C for 10 sec followed by 25 cycles of (denaturation at 96 °C for 10 sec, annealing at 50 °C for 5 sec and extension at 60 °C for 4 min) and the reaction was held at 4 °C to infinity.

3.8.2 Post Sequencing Purification and Sequence Reading

BioMag® beads were prepared by adding 1 ml of the BioMag® Carboxyl beads to the 1.5 ml Eppendorf tubes. The tubes were then placed in a magnetic separator for 2 min after which the supernatant was poured off and 0.5 ml EDTA was added and the beads re-suspended using a

pipette and then returned to the magnetic separator for 2 min. The supernatant was discarded and the beads re-suspended again in 0.5 ml of 0.5 M EDTA. After which, 19.5 ml of 5.0 M Sodium Chloride (NaCl) was then added to the beads and ready for use. BioMag® Carboxyl beads were well shaken to fully re-suspend after which 10 μ l of the cycle sequencing product was added and 42 μ l of 85% ethanol was added to the 10 μ l cycle sequenced product. This was followed by pipette mixing to homogenize the solution. The reaction plate was then placed onto SPRI Plate 96R ring magnetic plate for 5 min for the solution to become clear after separation from magnetic beads. The separated beads formed a circle at one corner of in the well. Whilst working on the magnet, 52 tubes were inverted onto a lint-free wipe via tapping gently on hard surface few times. After which, 200 μ l of 85% ethanol was dispensed into each well containing the beads. This was followed by a waiting time of 30 sec to allow the beads to settle before proceeding to the next step. Whilst working on the magnet, the plates were inverted onto a lint-free wipe by tapping it gently on a hard surface for few times to remove as much supernatant as possible as it possibly contained excess fluorescent dye and contaminants. Again, washing was repeated with the 200 μ l of the 85% ethanol as described previously. The plates were inverted onto a lint-free wipe, tapping it gently on a hard surface for five times to remove as much supernatant as possible as it possibly contained excess fluorescent dye and contaminants. Finally, whilst the plates were on the magnet, they were centrifuged at 500 rpm for 10 sec to evaporate all ethanol. Whilst working on the magnet, 40 μ l of DEPC treated water was added to the dry wells. The direct inject magnet was placed into the base of the sequencer alongside with the magnet. The sequencer was allowed to run for 24 h in ABI 3030xl Genetic analyzer.

3.8.3 Nucleotide Sequence Analysis

The raw sequences retrieved were inputted into Sequencher 5.0 software. The sequences were sorted according to quality and size. Sequences which were less than 250 bp and those that had quality less than 70% were deleted. The chromatograms of the individual contigs were perused to correct mismatches. Contigs were formed by aligning both forward and reverse sequences to generate near full length sequenced genes. Contigs formed were merged into one sequence file where they were converted into FASTA files. The sequences were subjected to the Basic Local Alignment Search Tool (BLAST) on the NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain and retrieve reference sequences similar to the sequenced genes. Sequenced genes were also genotyped using the RotaC v.2.0 automated online genotyping tool for group A rotaviruses.

3.9 Whole Genome Sequencing

3.9.1 Library Preparation for Next-Generation Sequencing

RNA fragmentation and priming

A volume of 5 μ l of purified dsRNA was added to 0.65 ml tube and 5 μ l of water was added to NTC-C control tube. Master mix was prepared by adding the reagents shown in Table A4. A volume of 5 μ l of master mix was added to each well containing the specific dsRNA to give a total volume of 10 μ l. The thermocycler was made to run by incubating the samples at 94 °C for 5 min after which the samples were transferred onto ice.

3.9.2 First strand cDNA synthesis

Master mix was prepared by adding the reagents listed in Table A5. The mixture was mixed by gently pipetting. Ten microliters (10 μ l) of the master mix was added to 10 μ l of the

fragmented and primed RNA template to make a total volume of 20 μ l. The samples were incubated in ABI preheated thermocycler as follows: Ten minutes at 25 °C, 30 min at 42 °C, 15 min at 70 °C, and held at 4 °C. Immediately after first strand synthesis, the second round reaction was prepared as below.

3.9.3 Second strand cDNA synthesis

Master mix was prepared by adding the reagents listed in Table A6 and mixed thoroughly by gentle pipetting and 60 μ l of the master mix was added to the first round product to give 80 μ l total volume. The mixture was incubated in a thermocycler for 1 h at 16 °C with the heated lid set at 40 °C.

Purification of fragmented amplicons

One hundred and forty four microliters (144 μ l) of resuspended AMPure XP beads was added to the second strand synthesized products, and was mixed well by pipette mixing and was incubated at room temperature for 5 min. The mixture was transferred from 0.65 PCR plates to 96 deep well plate. Two hundred microliters (200 μ l) of freshly prepared 80% ethanol was added to each well containing the samples while the plate was placed on a magnetic rack. The mixture was incubated on magnetic rag for 30 sec and the supernatant was carefully discarded. The washing was repeated once again by 200 μ l of freshly prepared 80% ethanol to each well containing the samples while the plate was placed on a magnetic rack and was incubated on magnetic rag for 30 sec and the supernatant carefully discarded. The beads were air dried while the plate was still on magnetic rack. The plate was removed from magnetic rack and 60 μ l 0.1 X TE buffer was added to the wells to elute the DNA from the beads. The beads were re-suspended in the elusion buffer by pipette mixing and then placed back on the magnetic rack until the solution became clear. Fifty-five and half microliters (55.5 μ l) of the supernatant was transferred to a clean 96 well PCR plate.

End Repair/ dA-tail of cDNA Library

Master mix was prepared by adding the reagents listed in Table A7 in 1.7 ml microtube 9.5 μ l of the master mix was added to the 55.5 μ l purified double stranded cDNA strand to give a total volume of 65 μ l in each well. The reaction was incubated as follows: 30 min at 20 °C, 30 min at 65 °C and held at 4 °C. After this the reaction was processed immediately for adaptor ligation.

3.9.4 Adapter ligation

The NEBNext Adaptor was retrieved from storage with a concentration of 15 μ M and was diluted with 10 mM NaCl to 1.5 μ l. Master mix was prepared in a 1.7 ml Eppendorf tube as listed in Table A8. A volume of 17.5 μ l of the master mix was added to the dA-Tailed cDNA and 1 μ l of diluted adaptor which made up the volume to 83.5 μ l in each well. This was followed by pipette mixing to and a quick spin to homogenize and flush down the mixture. The reaction was incubated in a thermocycler for 15 min at 20 °C. After the 15 min incubation period, 3 μ l of USER Enzyme was added to each well of the ligated product to make a total volume of 86.5 μ l per well and was proceeded by pipette mixing and brief spinning to homogenize and subsequently flush down the mixture. The ligated product was incubated at 37 °C for 15 min to denature the USER Enzyme after it had completed its task

3.9.5 Size selection of Adaptor ligated-DNA

A hundred microliters (100 μ l) of resuspended AMPure XP beads was added to the 100 μ l ligated product and was mixed well by pipette mixing for and was incubated at room temperature for 5 min. The mixture was transferred from 0.65 PCR plates to 96 well plates. A volume of 200 μ l of freshly prepared 80% ethanol was added to each well containing the samples while the plate was placed on a magnetic rack. The mixture was incubated on magnetic

rag for 30 sec and the supernatant was carefully discarded. The washing was repeated once again by adding 200 μ l of freshly prepared 80% ethanol to each well containing the samples while the plate was placed on a magnetic rack and was incubated on magnetic rag for 30 sec and the supernatant carefully discarded. The beads were air dried while the plate was still on magnetic rack. The plate was removed from magnetic rack and 22 μ l of 0.1 X TE buffer was added to the wells to elute the DNA from the beads. Twenty microliters (20 μ l) of the supernatant was transferred to a clean 96 well PCR plate.

3.9.6 PCR Library Enrichment

Master mix was prepared as detailed in Table A9 (refer to the appendix) in a 1.7 ml Eppendorf tube. Twenty-four indices (10 μ M) for 24 samples were retrieved from storage and 2.5 μ l of specific primers were added to the wells containing 27.5 μ l purified adaptor ligated cDNA such that each well received a particular index primer to make a total volume of 50 μ l in each well. The mixture was vortex and briefly span to mix and flush down the mixture. The PCR reaction was set as follows: initial denaturation at 98 °C for 30 sec for 1 cycle followed by 15 cycles of (denaturation at 98 °C for 30 sec, annealing/extension at 65 °C for 75 sec and a final extension at 65 °C for 75 sec) and held at 4 °C.

Purification of PCR Reaction

Forty-five milliliters (45 ml) of re-suspended AMPure XP beads was added to the PCR enriched products and was mixed well by pipette mixing for and was incubated at room temperature for 5 min. The mixture was transferred from 0.65 PCR plates to 96 deep well plate. Two hundred microliters (200 μ l) of freshly prepared 80% ethanol was added to each well containing the samples while the plate was placed on a magnetic rack. The mixture was incubated on magnetic rag for 30 sec and the supernatant was carefully discarded. The washing was repeated once again by 200 μ l of freshly prepared 80% ethanol to each well containing the

samples while the plate was placed on a magnetic rack and was incubated on magnetic rag for 30 sec and the supernatant carefully discarded. The beads were air dried while the plate was still on magnetic rack. The plate was removed from magnetic rack and 23 μ l of 0.1 X TE buffer was added to the wells to elute the DNA from the beads. The beads were re-suspended in the elusion buffer by pipette mixing and then placed back on the magnetic rack until the solution became clear. Twenty microliters (20 μ l) of the supernatant was transferred to a clean 96 well PCR plate.

3.9.7 Normalization of DNA library

Twenty-four Qubit tubes (Thermofisher Scientific, U.S.A. & Waltham) were labeled for samples and controls while the Qubit working solution was diluted for 30 sample preparation by adding 30 μ l of Qubit reagent to 5,970 μ l of Qubit buffer and 190 μ l of the working solution was pipetted into the microtubes.

Ten microliters (10 μ l) of the Qubit standard 1 was added to labeled Qubit Eppendorf tubes containing 190 μ l of Qubit working solution and was vortex briefly to mix. Ten microliters (10 μ l) of Qubit standard 2 was added to labeled standard 2 Qubit Eppendorf tubes containing 190 μ l of Qubit working solution and was vortexed briefly to mix.

The tubes were allowed to incubate for 2 min at room temperature. The samples were then loaded on the Qubit fluorimeter and the readings were taken. The concentration of the sample was determined and tabulated Table A10.

3.9.8 Denaturation of DNA libraries and PhiX control Prior to Sequencing with MiSeq

Illumina

The DNA library was pooled together by adding 5 μ l of sample from each well into a single Eppendorf tube and then vortexed and span briefly. Five microliters (5 μ l) of the well mixed

pooled library was pipetted into each Eppendorf tube and 10 μ l of 10 N NaOH was added and 800 μ l of nuclease free water was added, followed by short vortexing and spinning and 5 min room temperature incubation to denature DNA. Nine hundred and sixty microliters (960 μ l) of chilled HT1 buffer was added to the denatured DNA. In order to denature the PhiX control, 5 μ l of 2 nM PhiX was added to in a separate eppendorf tube and 5 μ l of freshly prepared 0.2 nM NaOH was added. The mixture was briefly vortex and spin and incubated for 5 min at room temperature to denature the DNA into single strands. Nine hundred and ninety (990 μ l) of chilled HT1 buffer was added to the denatured PhiX DNA to give 10 pM solution. For the spiking of 20% PhiX, 480 μ l of the denatured template library was placed in a separate eppendorf tube and 120 μ l of the 10 pM denatured PhiX was added. For the spiking of 5% PhiX, 570 μ l of the denatured template library was placed in a separate eppendorf tube and 30 μ l of the 10 pM denatured PhiX was added. Six hundred microliters (600 μ l) of 12 pM denatured library spiked with PhiX was loaded onto the Miseq reagent cartridge and was made to run for 72 h.

3.10 Whole genome phylogenetic analysis

The genotypes for the various gene segments were determined by RotaC version 2.0. Multiple sequence alignment was made for each gene using MUSCLE algorithm implemented in MEGA 6.0. Maximum likelihood trees were constructed for each gene segment in PhyML 4.0 using the optimal model. Approximate likelihood ratio test statistics were computed for branch support and the best model selection was done based on Akaike information criterion which was general time reverse. Sub genetic clusters were also computed and sequences tested for possible genetic recombination using the Genetic Algorithm Recombination Detection. Selection analysis was performed using a combination of Single Likelihood Ancestor Counting, Fixed Effect Likelihood, and Random Effect Likelihood analysis.

CHAPTER FOUR

4.0 RESULTS

This study obtained 36 stool samples from children under the age of 5 years who reported with diarrhea at three Health Centers in Breman Asikuma. Rotavirus antigen screening was conducted using the Proflow™ kit to detect the presence of rotaviruses. RNA extraction was carried out to obtain pure and highly concentrated rotavirus RNA. The RNAs were then resolved on a Polyacrylamide gel electrophoresis (PAGE). RT-PCR was conducted to genotype the isolated viruses.

4.1 Rotavirus Prevalence in Breman Asikuma

Rotavirus prevalence of 8.3% was recorded for this study. Of the 36 diarrheic stools examined, 3 children were found to be shedding the virus. These were confirmed by Polyacrylamide gel electrophoresis (PAGE) where all 3 samples (100%) yielded electrophoretic patterns typical of rotavirus (Fig 4.1). Two of the three rotavirus positive samples (2/3; 66.7%) had the classical long rotavirus RNA electrophoretic pattern whilst one (1/3; 33.3%) exhibited the classical short RNA profile (Table 4.1).

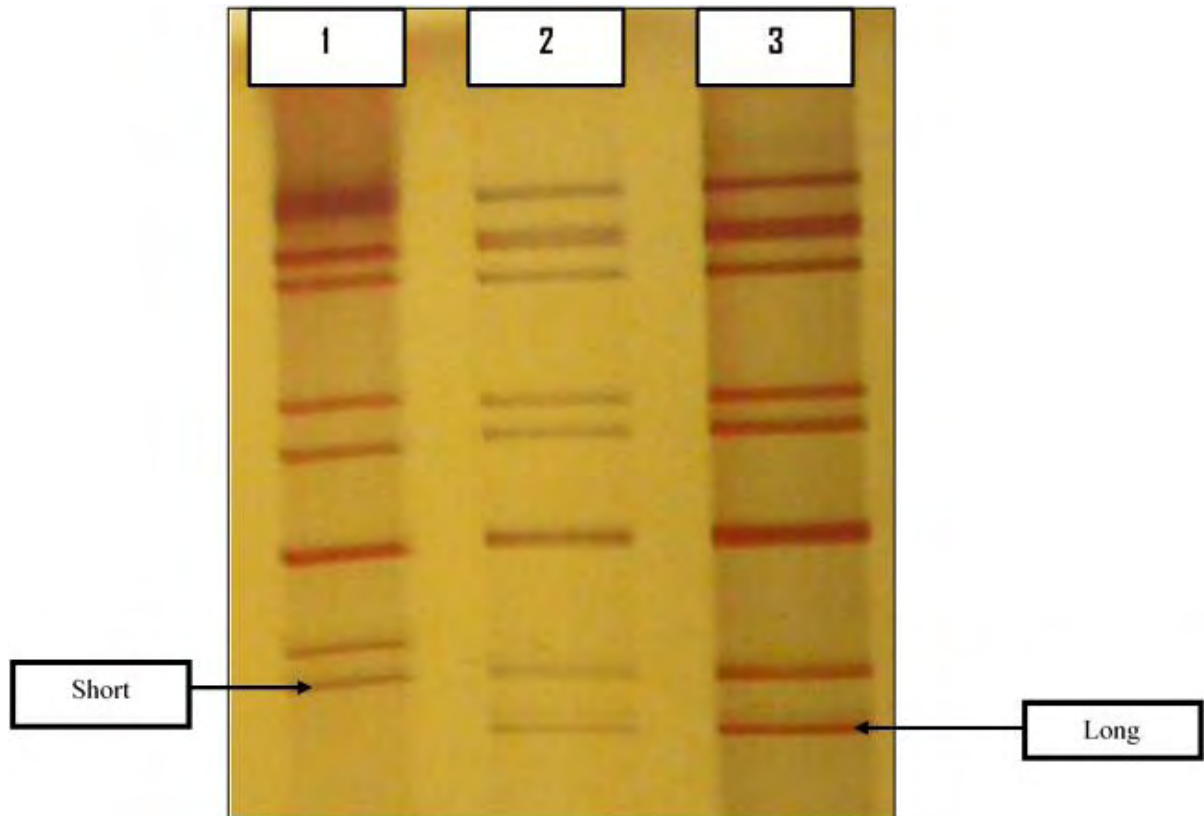


Figure 4.1: Electrophoretotypes of Representative Rotavirus Strains analyzed in this study (positive samples) showing the distinct rotavirus migration pattern. Lane 1 shows the short electrophoretic migration pattern; Lane 2 and 3 show the long electrophoretic migration pattern. The figure shows the gene segments of the rotavirus.

Table 4.1: PAGE distribution of human rotavirus strain in Breman Asikuma

Electrophoretotypes	Total	Percentage (%)
Long	2	66.7
Short	1	33.3
Total	3	100

4.2 Genotype Analysis

4.2.1 Circulating VP7 and VP4 Genotypes in Breman Asikuma

Viral RNA extracts of all positive samples (BA4, BA5 and BA12) were reverse transcribed and their VP7 and VP4 genes amplified using gene specific primers. After electrophoresing on agarose gels all three samples showed positive amplification of both VP7 and VP4 genes with amplicon sizes of 1062 and 876 base pairs respectively (Fig 4.2). Genotyping of the amplified genes was done using genotype specific primers. The VP4 genes from samples BA5 and BA12 possessed the P[8] genotype whilst that of sample BA4 was genotyped as P[6] (Fig 4.3). No mixed infections were recorded. Similarly, the VP7 genes of rotavirus strains from samples BA5 and BA12 were genotyped as G9 whilst VP7 gene of rotavirus strain from sample BA4 was genotyped as G3 (Fig 4.4).

4.2.2 Rotavirus Strains Circulating among Children in Breman Asikuma

Two distinct strains were identified with the common G and P genotype combinations being strains bearing the genotype G9P[8] and G3P[6]. Rotavirus G9P[8] was the commonly circulating strain.

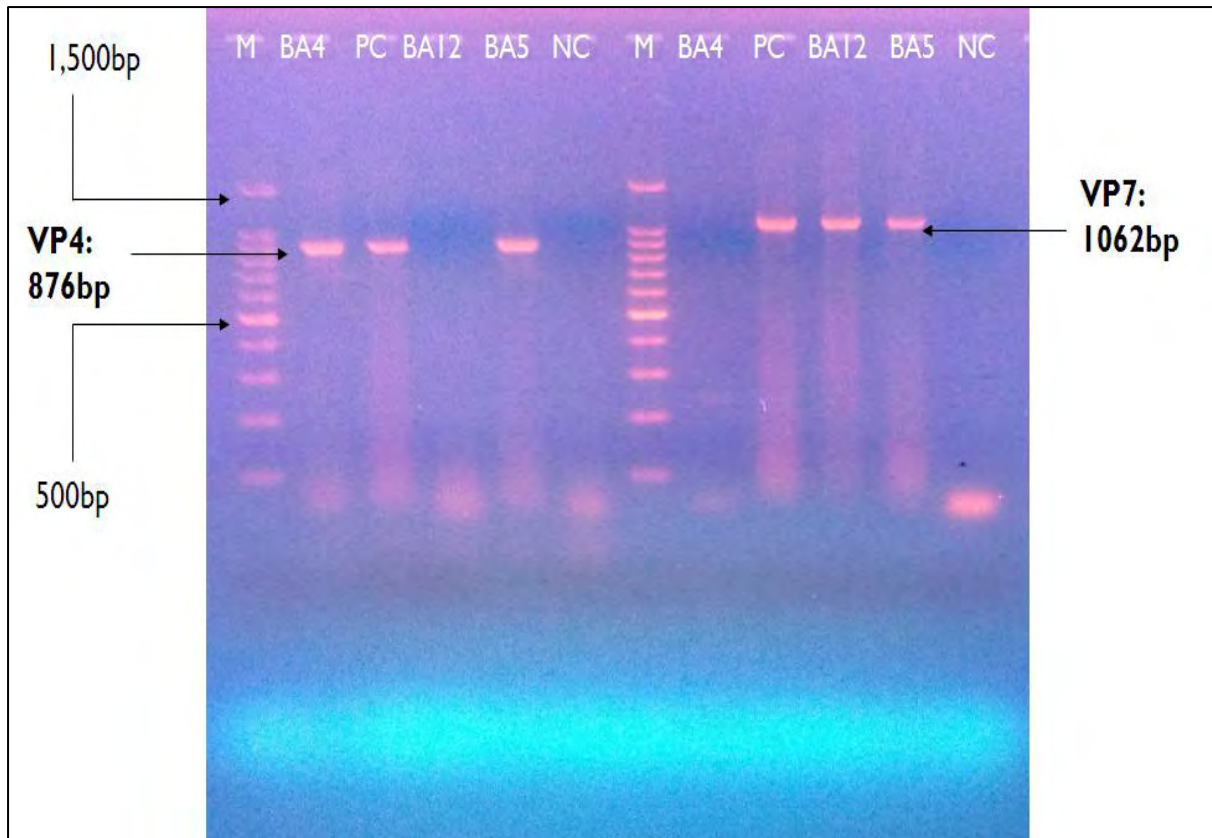


Figure 4.2: 1.5% Agarose gel of PCR amplicons of VP7 & VP4 genes of study samples. Lanes 2 and 5 show successfully amplified VP4 genes; Lanes 10 and 11 show successfully amplified VP7 genes; Lanes 1 and 7: 100 bp marker; Lanes 3 and 9: VP4 and VP7 Positive Controls (PC); Lanes 6 and 12: Negative Control (NC). The counting of the lanes start from left to right starting with M been lane 1 and NC been lane 12.

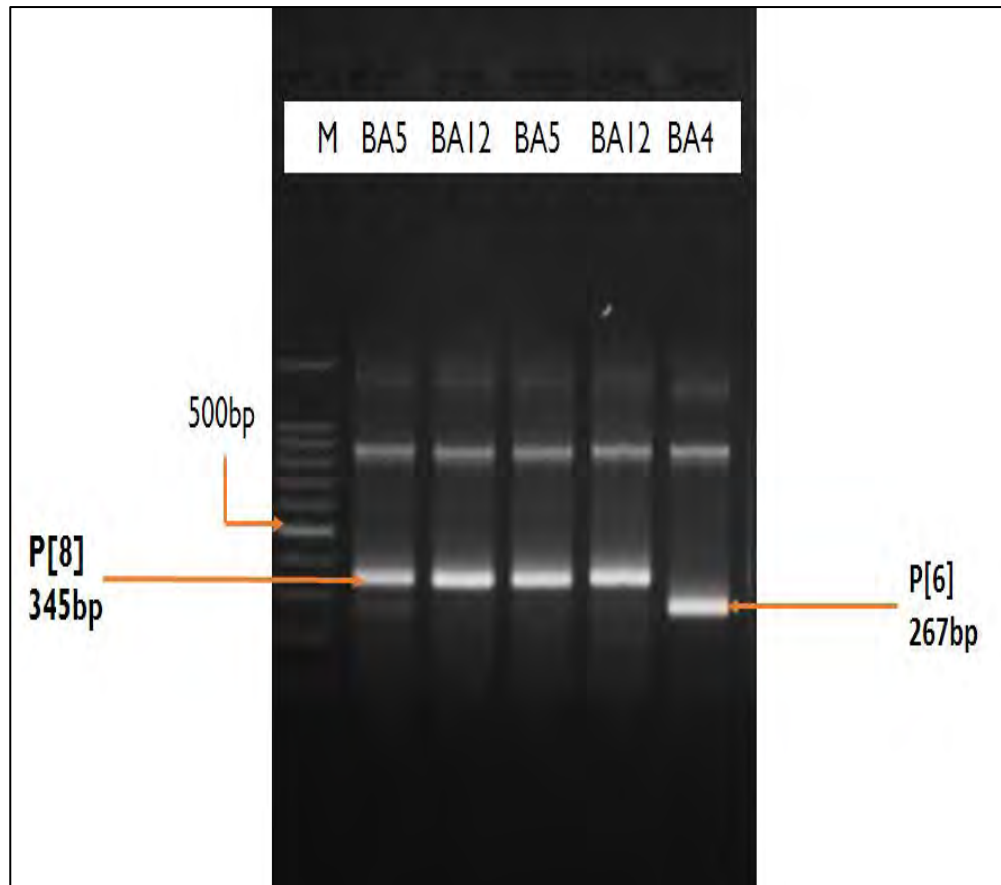


Figure 4.3: 1.5% Agarose gel of PCR amplicons of VP4 genotypes of study samples. Lanes 2 to 5 show amplified VP4 genotypes of varying band sizes; Lane 1: 100 bp marker. The counting of the lanes start from left to right starting with “M” been lane 1 and “BA4” been lane 6.

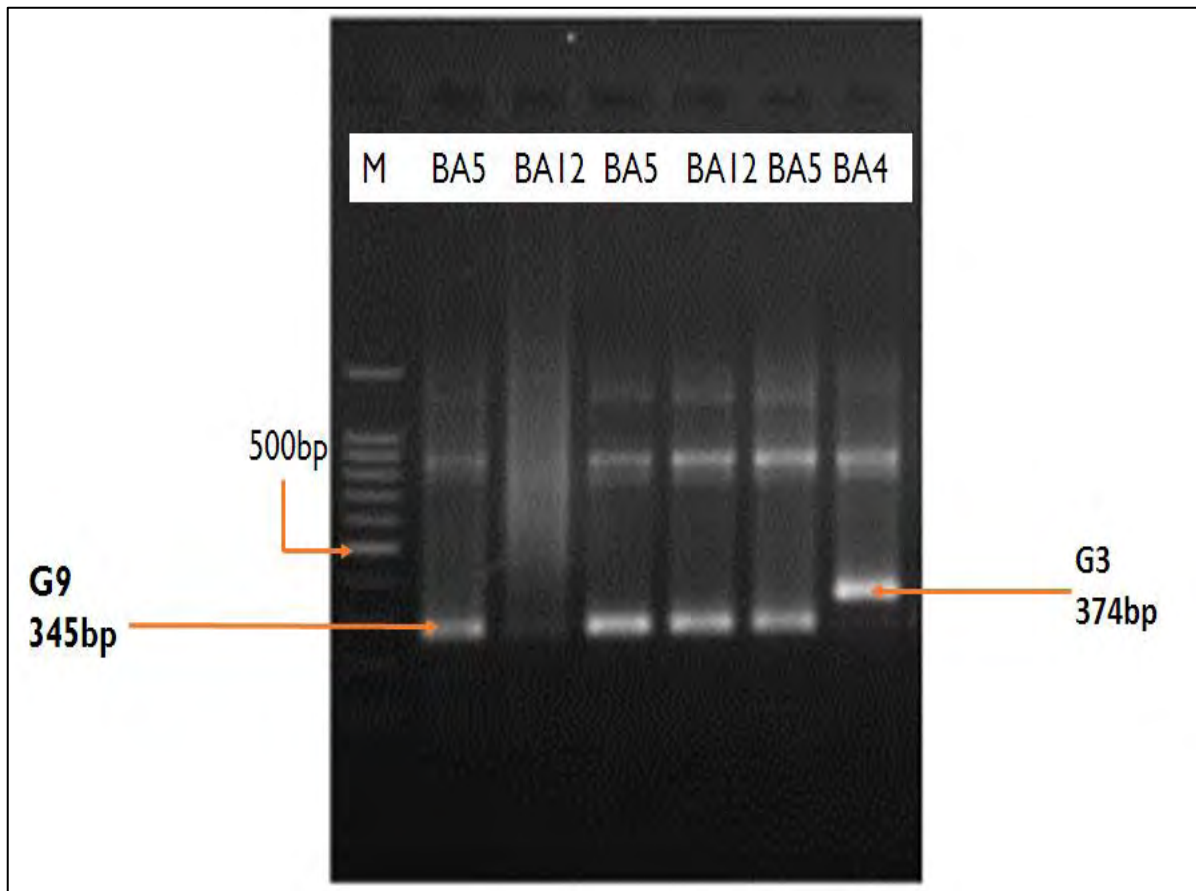


Figure 4.4: 1.5% Agarose gel of PCR amplicons of VP7 genotypes of study samples. Lanes 2 to 6 show amplified VP7 genotypes of varying band sizes; Lane 1: 100 bp marker. The counting of the lanes start from left to right starting with “M” been lane 1 and “BA4” been lane 7.

4.3 Phylogenetic analysis

4.3.1 Phylogenetic analysis of study VP7 gene of G3 genotype

Phylogenetic analysis of study G3-VP7 gene showed it was not unusual when compared to strains elsewhere bearing the same G3 genotypes. However, further, observation showed the G3-VP7 gene clustering with other globally circulating G3 strains detected in the late 2000's (Fig 4.5). Interestingly, the study G3 rotavirus strain clustered in Lineage I away from other Ghanaian G3 strains also detected in the late 2000's which clustered within Lineage II as shown in the phylogenetic dendrogram . The study strain RVA/Hu/BA4/2015/G3P6 was found to

cluster closely with a Nigerian G3P6 isolate (RVA/Hu/NGR/NGR02/2013/G3P6) showing nucleotide sequence similarity of 89% with each other

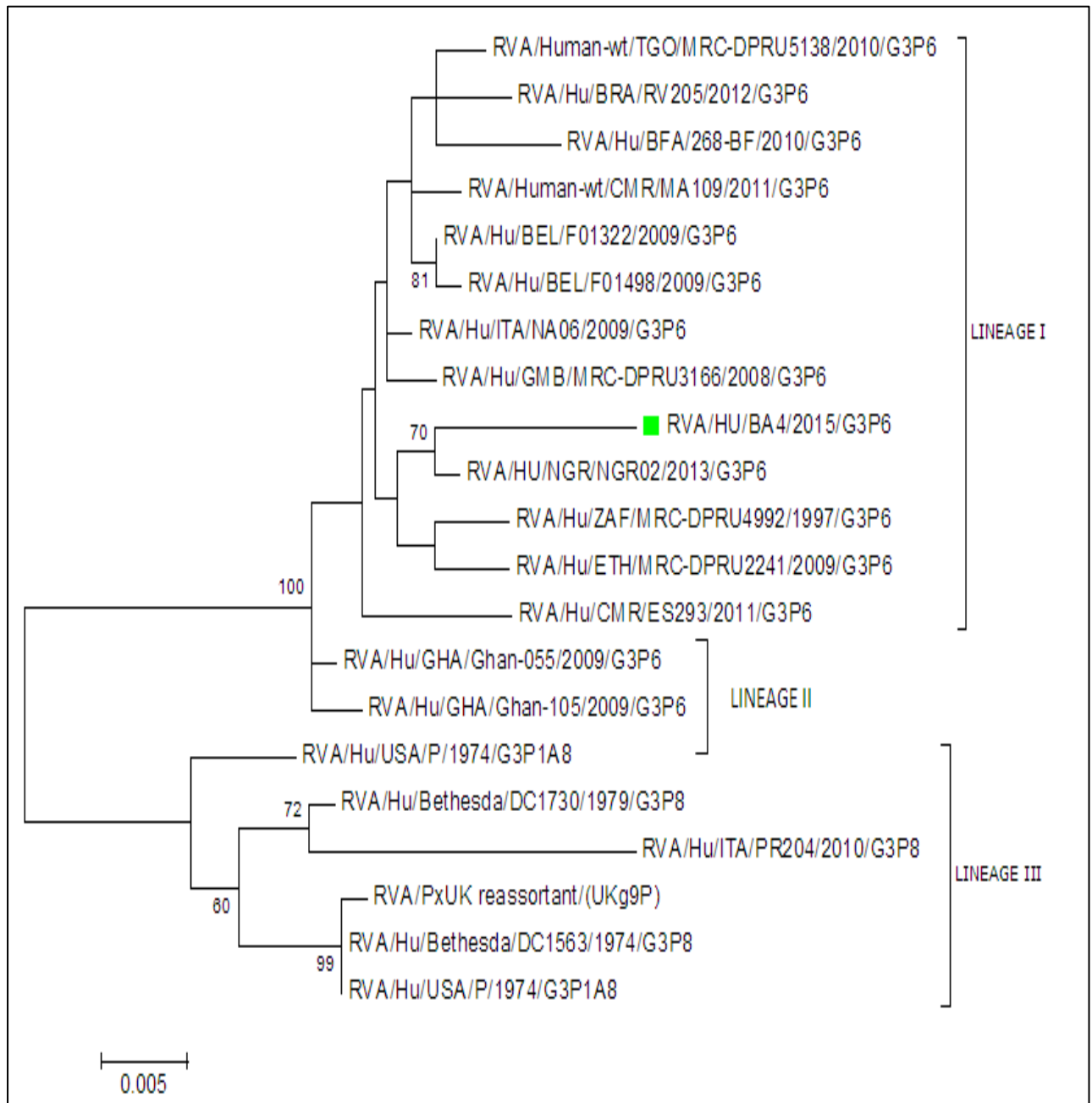


Figure 4.5: Phylogenetic dendrogram of VP7 gene of G3 rotavirus strains. The phylogenetic analysis included the VP7 nucleotide sequence of Ghanaian study strain (indicated in green circle) and other published African, Asian and European G3 VP7 nucleotide sequences of human origin. Maximum likelihood phylogenetic analysis was performed using the Kimura 2-parameter method in the Mega7.0.26 software package; significant bootstrap values (1000 replicates) of $\geq 70\%$ are indicated at each node. Scale bar indicates genetic distance expressed as number of nucleotide substitutions per site.

4.3.2 Phylogenetic analysis of study VP7 genes of G9 genotype

The detected study G9 rotavirus strains formed a monophyletic cluster away from other reference strains (Fig 4.6). The open reading frames of the VP7 gene segments of the two G9 study strains RVA/Hu/GHA/BA5/2015/G9P8 and RVA/Hu/GHA/BA12/2015/G9P8 shared nucleotide identity >93% among themselves and just between 89% and 72% with other reference strains. They, however, shared the closest relation with published strain(s) RVA/Hu/PAK100/2010/G9P[6] with nucleotide sequence identity of 76.7%.

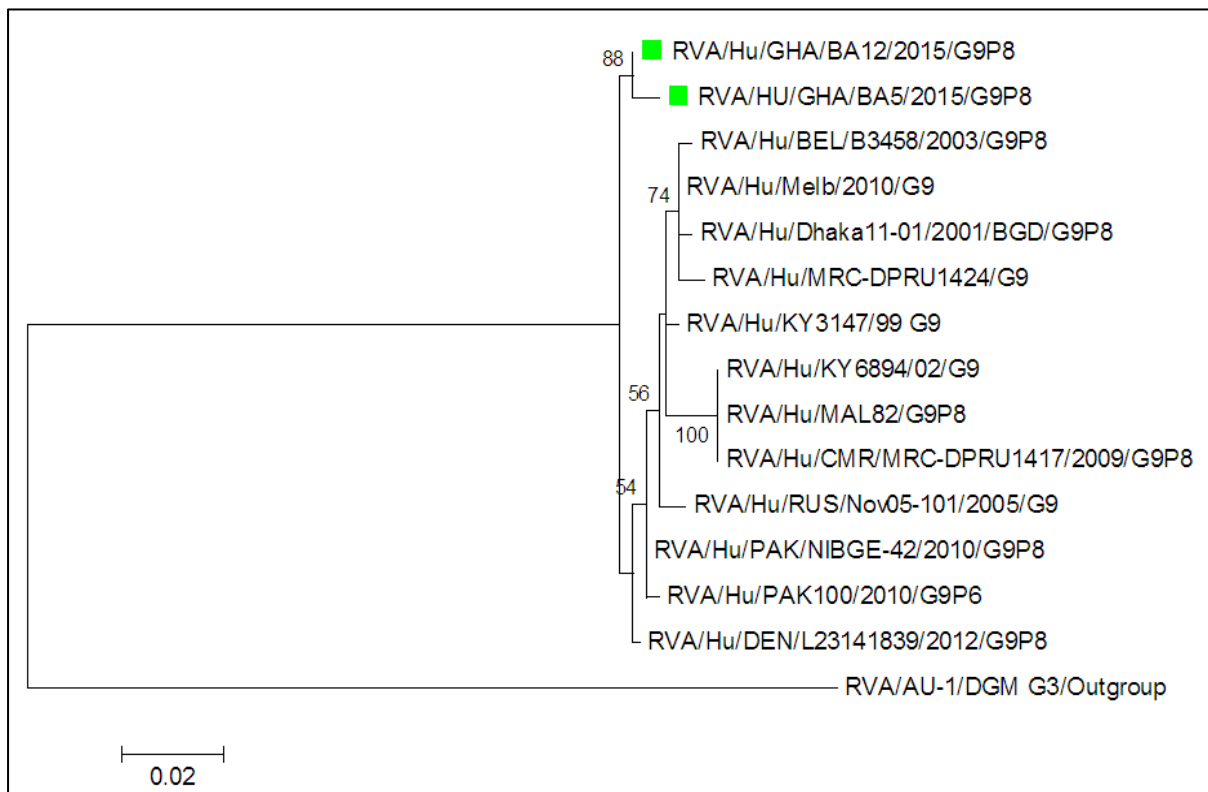


Figure 4.6: Phylogenetic dendrogram of VP7 gene of G9 rotavirus strains. The phylogenetic analysis included the VP7 nucleotide sequence of Ghanaian study strain (indicated in green squares) and other published African, Asian and European G9 VP7 nucleotide sequences of human origin. Maximum Likelihood phylogenetic analysis was performed using the Kimura 2-parameter method in the Mega7.0.26 software package; significant bootstrap values (1000 replicates) of $\geq 70\%$ are indicated at each node. Scale bar indicates genetic distance expressed as a number of nucleotide substitutions per site.

4.3.3 Phylogenetic analysis of study VP4 gene of P[6] genotype

The VP4 gene of the study P[6] strain clustered in Lineage II along with globally circulating strains from Africa, Asia and Europe as given in (Fig 4.7). Similar to the VP7 genes, nucleotide sequences of the VP4 gene of the study P[6] (RVA/Hu/BA4/2015/P6) strain clustered away from other published Ghanaian VP4 genes of P[6] (RVA/Hu-wt/GHA/Ghan-149/2008/P6, RVA/Hu-wt/GHA/GH018-08/2008/P6, RVA/Hu-wt/GHA/Ghan-105/2009/P6 and RVA/Hu-wt/GHA/MRC-DPRU1818/1999/P6) strains isolated between 1999 and 2008 clustering within the Lineage I. The study strain however clustered closely with a Gambian P[6] strain isolated in the year 2008 sharing nucleotide sequence identity of 92%.

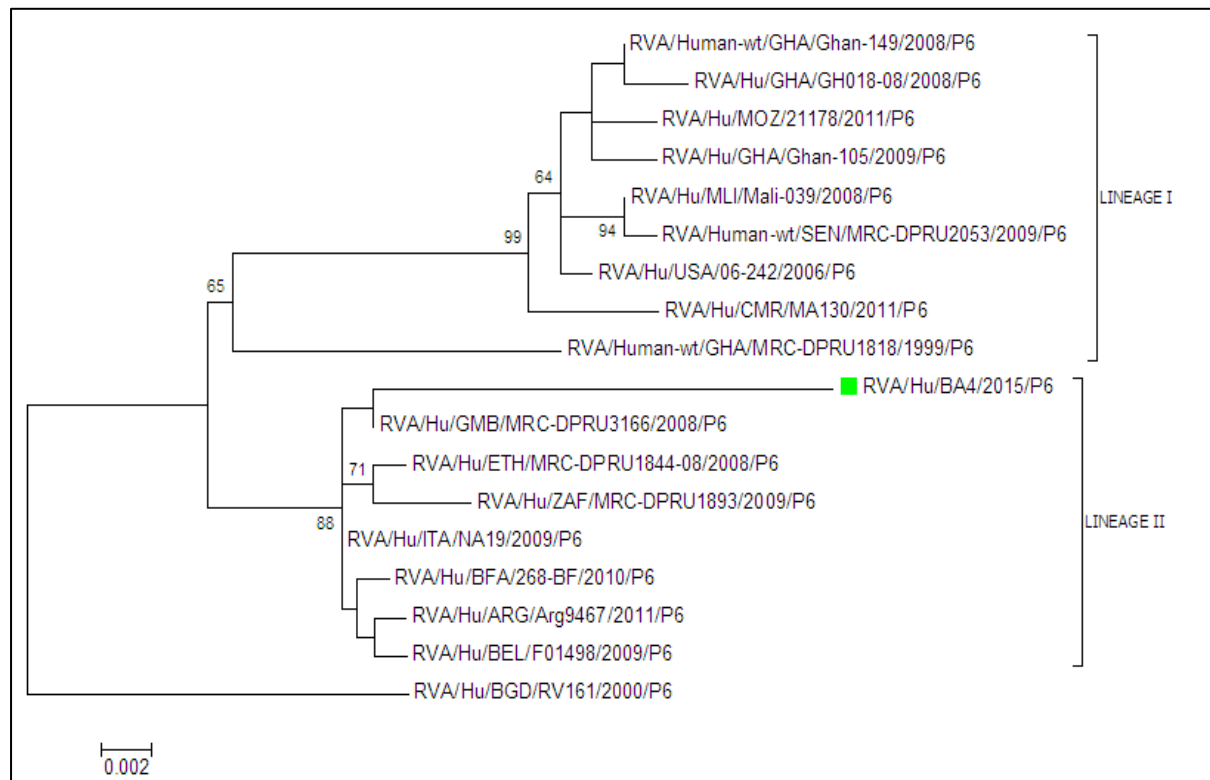


Figure 4.7: Phylogenetic dendrogram of VP4 gene of P[6] rotavirus strains. The phylogenetic analysis included the VP4 nucleotide sequence of Ghanaian study strain (indicated in green square) and other published African, Asian and European P[6] VP4 nucleotide sequences of human origin. Maximum Likelihood phylogenetic analysis was performed using the Kimura 2-parameter method in the Mega7.0.26 software package; significant bootstrap values (1000 replicates) of $\geq 70\%$ are indicated at each node. Scale bar indicates genetic distance expressed as number of nucleotide substitutions

4.3.4 Phylogenetic analysis of study VP4 genes of P[8] genotype

Nucleotide sequences of P[8]-VP4 genes of two of the Ghanaian study strains (RVA/Hu/GHA/BA5/2015/G9P[8] and RVA/Hu/GHA/BA12/2015/G9P[8]) were found to be closely related with each other sharing nucleotide sequence identity of 93%. Phylogenetic analysis of nucleotide sequences of these study VP4 gene segments showed them grouping closely together and with other published African, Asian and European strains also of bearing the P[8] genotype (Fig 4.8). These study strains also shared nucleotide sequence similarities between 92% and 64% with the globally circulating reference strains.



Figure 4.8: Phylogenetic tree of VP4 gene of P[8] rotavirus strains. The phylogenetic analysis included the VP4 nucleotide sequence of Ghanaian study strains (indicated in green square) and other published African, Asian and European P[8] VP4 nucleotide sequences of human origin. Maximum Likelihood phylogenetic analysis was performed using the Kimura 2-parameter method in the Mega7.0.26 software package; significant bootstrap values (1000 replicates) of $\geq 70\%$ are indicated at each node. Scale bar indicates genetic distance expressed as number of nucleotides

4.4 Whole genome constellation of rotavirus G9P[8] study strains

Using the RotaC automated genotyping tool for group A rotaviruses, each gene (VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NP2-NSP3-NSP4-NSP5) was assigned a genotype on the basis of nucleotide sequence identity analysis of the near full-length sequences of all gene segments of study strains. Study samples RVA/Hu/GHA/BA5/2015/G9P[8] and RVA /Hu/GHA/BA12 /2015/G9P[8] shared a Wa-Like genotype constellation (G9-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) with a genotype one backbone (Table 4.2).

Table 4.2: Whole Genome constellation of rotavirus G9P[8] study strains

Strains	Gene Segments										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Hu/GHA/BA5/2015/G9P[8]	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Hu/GHA/BA12/2015/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/HU/ITA/AST123/2007/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu/BEL/BEL/BE0003/2004/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu-tc/USA/W161/2009/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu-tc/BGD/MMC38/2005/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu/CMR/DPRU1723/2009/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu/ZAF/DPRU4677/2010/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1

NB. The sequences represented on this table all have Wa-like backbones. The strains in bold are samples from the current study. Both belong to the Wa-like genogroup with no reassortments.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION, LIMITATIONS, AND RECOMMENDATIONS

5.1 Discussion

Rotavirus has been observed to be the single most pertinent pathogen connected with diarrhea cases in both hospital admitted and out patients less than five years of age (Alkali et al., 2015). In Ghana previous studies on rotavirus, infection in children have been conducted in both rural and urban settings (Armah et al., 2010; Armah et al., 1994; Armah et al., 2001; Asmah et al., 2001; Binka et al., 2011; Enweronu-Laryea et al., 2012) but very few in a semi-urban community. This study was aimed at determining the prevalence and circulating genotypes of rotavirus strains in children under five years of age with acute gastroenteritis in a semi-urban community such as Asikuma Breman found in the Central region of Ghana.

A total of 36 samples collected during the period of September/2015 to August/2016 were analyzed. The study showed a prevalence of 8.3%. There have been reports of a global decline in rotavirus associated diarrheal diseases in children less than five years following the introduction of rotavirus vaccination into the immunization programs of several countries (Tate et al., 2016). Ghana is no exception and introduced rotavirus vaccination into its Expanded National Immunization program (EPI) in April, 2012. Since then, rotavirus prevalence studies carried out in the Greater Accra region of Ghana reported a decline in rotavirus associated diarrhea disease from 50% pre-vaccine era to 18% post vaccine era (Enweronu-Laryea et al., 2014a)

Similar reports were seen in other countries such as Zambia, which reported a decline in RVA prevalence from 40.1% pre-vaccine period to 30.1% and 26.2% prevalence in the first and second years post vaccination era (Mpabalwani et al., 2016). Similarly, RVA surveillance

studies in Morocco, published by Benhafid et al. (2015), reported 44% RVA prevalence in the pre-vaccine era to 29%, 15% and 24% prevalence in the first, second and third years post vaccination. In addition, a study conducted in Georgia Atlanta reported 30% RVA prevalence in the pre-vaccine era to 13% post vaccination period (Anderson & Sederdahl, 2014). An RVA study conducted among Malawian children reported 32% prevalence pre-vaccine to 24% RVA prevalence 4 years post vaccine introduction in Malawi (Bar-Zeev et al., 2016).

All these previous studies cited suggest a reduction in the prevalence of rotavirus in the post vaccine era. A prevalence rate of 8.3% recorded in this study is accounted for by the implementation of rotavirus vaccination in this region, which had over 95% vaccination coverage. Moreover, Breman Asikuma is not a densely populated community and thus a possible reduction in the rate of transmission of the virus amongst children.

Rotavirus G3P[6] and G9P[8] were the two important rotavirus strains associated with diarrhea infections in children under five years of age. A pre-vaccine era study found G3 as the most prevalent strain (78%) in Ghana (Asmah et al., 2001). Similarly, the prevalence of G3 strains in Ghana was reported (Enweronu-Laryea et al., 2013). Among human rotaviruses, G9 has emerged as the fifth most important genotype circulating globally (Ribas Mde et al., 2015b).

The emergence of rotavirus G9P[8] strain has been reported globally. In this study, G9 was the most predominant genotype. Studies conducted in Ghana, Cuba, Wuhan in China and Detroit in the USA reported the emergence and predominance of rotavirus G9 genotype (Abdel-Haq et al., 2011; Armah et al., 2003; Ribas Mde et al., 2015a; Yang et al., 2007).

Rotaviruses possess a double stranded RNA and hence have the ability to undergo genetic reassortment. Since vaccines currently in use are live attenuated, there is the probability of the vaccine strain reverting to wild type rotavirus or the wild type reassorting with the vaccine strains (Estes & Greenberg, 2013). Full genome classification system was developed for group

A rotaviruses in 2008, by the Rotavirus Classification Working Group (RCWG) to increase our chances of detecting reassortant strains using the whole viral genome (Matthijnssens et al., 2008). This system of classification assigns a specific genotype to each of the 11 gene segments of a particular RVA strain according to the established nucleotide percent cut-off value. Applying this approach, the genome of individual rotavirus strains are given the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx representing the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 encoding gene segments, respectively (Matthijnssens et al., 2011). Commonly detected rotavirus genotype constellations associated with childhood diarrhea include the human Wa-like (G1/G3/G4/G9-P[8]-I1-C1-M1-A1-N1-T1-E1-H1), human DS-1-like (G2/G8-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) and the human AU-1-like (G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3) strains (Matthijnssens et al., 2012b). This study reported the human Wa-like which is responsible for the diarrhea in the patients.

Phylogenetic studies of the VP7 and VP4 genes of the two commonly circulating rotavirus strains in this semi-urban community revealed they were not unusual when compared to other globally circulating G3P[6] and G9P[8] rotavirus strains unlike previous studies that have reported rotavirus vaccine strains in diarrheic children during surveillance (Desselberger, 2014). This study found no genetic relatedness between strains detected and rotavirus vaccine strains. Complete genome analysis of study G9P[8] rotavirus strains revealed them to have the human Wa-like genotype constellation (G9-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) with a genotype one backbone similar to other globally circulating rotavirus G9P[8] strains (Table 4.2). No intra or inter genogroup reassortment was however observed in this study.

5.2 Conclusions

G9P[8] and G3P[6] strains were the commonly detected and circulating rotavirus strains during the period of study. Phylogenetic analysis of the detected study G9P[8] and G3P[6] strains showed their VP7/VP4 gene segments were not unusual when compared with other globally circulating rotavirus G9P[8] and G3P[6] strains. Full genome analysis also revealed the study G9P[8] strains to have the human Wa-Like genotype constellation with no reassortment events occurring within their gene segments.

5.3 Limitations

The study was carried out within one rotavirus study period, thus a short duration of time to fully understand the pattern of circulating rotavirus strains within the study area in the post vaccine era. In addition, the sample size was small relative to most studies due to the lack of diarrhea cases in the study area.

5.4 Recommendations

To maintain the surveillance in the study area for longer periods to increase the sample size and also be able to fully describe and understand the effect of the vaccine. Full genome classification is recommended for all isolated rotavirus samples to determine genetic recombination and reassortment events.

REFERENCES

- Abbott, C., Tiede, B., Armah, G., & Mahmoud, A. (2012). Evaluation of cost-effectiveness of live oral pentavalent reassortant rotavirus vaccine introduction in Ghana. *Vaccine*, *30*(15), 2582-2587. doi: 10.1016/j.vaccine.2012.01.076
- Abdel-Haq, N., Amjad, M., McGrath, E., Chearskul, P., Amer, A., Salimnia, H., et al. (2011). Emergence of human rotavirus genotype G9 in metropolitan Detroit between 2007 and 2009. *J Med Microbiol*, *60*(Pt 6), 761-767. doi: 10.1099/jmm.0.026807-0
- Affranchino, J. L., & Gonzalez, S. A. (1997). Deletion mapping of functional domains in the rotavirus capsid protein VP6. *J Gen Virol*, *78* (Pt 8), 1949-1955. doi: 10.1099/0022-1317-78-8-1949
- Afrikanova, I., Miozzo, M. C., Giambiagi, S., & Burrone, O. (1996). Phosphorylation generates different forms of rotavirus NSP5. *J Gen Virol*, *77* (Pt 9), 2059-2065. doi: 10.1099/0022-1317-77-9-2059
- Alkali, B. R., Daneji, A. I., Magaji, A. A., & Bilbis, L. S. (2015). Clinical Symptoms of Human Rotavirus Infection Observed in Children in Sokoto, Nigeria. *Adv Virol*, *2015*, 890957. doi: 10.1155/2015/890957
- American Academy of Pediatrics Committee on Infectious, D. (2007). Prevention of rotavirus disease: guidelines for use of rotavirus vaccine. *Pediatrics*, *119*(1), 171-182. doi: 10.1542/peds.2006-3134
- Anderson, E. J., & Sederdahl, B. K. (2014). Intussusception risk increased after rotavirus vaccination but outweighed by benefits. *Evid Based Med*, *19*(5), 191-192. doi: 10.1136/eb-2014-101793
- Aoki, S. T., Settembre, E. C., Trask, S. D., Greenberg, H. B., Harrison, S. C., & Dormitzer, P. R. (2009). Structure of rotavirus outer-layer protein VP7 bound with a neutralizing Fab. *Science*, *324*(5933), 1444-1447. doi: 10.1126/science.1170481

- Arias, C. F., Romero, P., Alvarez, V., & Lopez, S. (1996). Trypsin activation pathway of rotavirus infectivity. *J Virol*, *70*(9), 5832-5839.
- Armah, G., Lewis, K. D., Cortese, M. M., Parashar, U. D., Ansah, A., Gazley, L., et al. (2016). A Randomized, Controlled Trial of the Impact of Alternative Dosing Schedules on the Immune Response to Human Rotavirus Vaccine in Rural Ghanaian Infants. *J Infect Dis*, *213*(11), 1678-1685. doi: 10.1093/infdis/jiw023
- Armah, G. E., Hoshino, Y., Santos, N., Binka, F., Damanka, S., Adjei, R., et al. (2010). The global spread of rotavirus G10 strains: Detection in Ghanaian children hospitalized with diarrhea. *J Infect Dis*, *202 Suppl*, S231-238. doi: 10.1086/653572
- Armah, G. E., Kapikian, A. Z., Vesikari, T., Cunliffe, N., Jacobson, R. M., Burlington, D. B., et al. (2013). Efficacy, immunogenicity, and safety of two doses of a tetravalent rotavirus vaccine RRV-TV in Ghana with the first dose administered during the neonatal period. *J Infect Dis*, *208*(3), 423-431. doi: 10.1093/infdis/jit174
- Armah, G. E., Mingle, J. A., Dodoo, A. K., Anyanful, A., Antwi, R., Commey, J., et al. (1994). Seasonality of rotavirus infection in Ghana. *Ann Trop Paediatr*, *14*(3), 223-229.
- Armah, G. E., Pager, C. T., Asmah, R. H., Anto, F. R., Oduro, A. R., Binka, F., et al. (2001). Prevalence of unusual human rotavirus strains in Ghanaian children. *J Med Virol*, *63*(1), 67-71.
- Armah, G. E., Steele, A. D., Binka, F. N., Esona, M. D., Asmah, R. H., Anto, F., et al. (2003). Changing patterns of rotavirus genotypes in Ghana: emergence of human rotavirus G9 as a major cause of diarrhea in children. *J Clin Microbiol*, *41*(6), 2317-2322.
- Arnold, M. M., & Patton, J. T. (2011). Diversity of interferon antagonist activities mediated by NSP1 proteins of different rotavirus strains. *J Virol*, *85*(5), 1970-1979. doi: 10.1128/JVI.01801-10

- Asmah, R. H., Green, J., Armah, G. E., Gallimore, C. I., Gray, J. J., Iturriza-Gomara, M., et al. (2001). Rotavirus G and P genotypes in rural Ghana. *J Clin Microbiol*, 39(5), 1981-1984. doi: 10.1128/JCM.39.5.1981-1984.2001
- Bar-Zeev, N., Jere, K. C., Bennett, A., Pollock, L., Tate, J. E., Nakagomi, O., et al. (2016). Population Impact and Effectiveness of Monovalent Rotavirus Vaccination in Urban Malawian Children 3 Years After Vaccine Introduction: Ecological and Case-Control Analyses. *Clin Infect Dis*, 62 Suppl 2, S213-219. doi: 10.1093/cid/civ1183
- Barnes, G. L., Lund, J. S., Adams, L., Mora, A., Mitchell, S. V., Caples, A., et al. (1997). Phase 1 trial of a candidate rotavirus vaccine (RV3) derived from a human neonate. *J Paediatr Child Health*, 33(4), 300-304.
- Barnes, G. L., Lund, J. S., Mitchell, S. V., De Bruyn, L., Piggford, L., Smith, A. L., et al. (2002). Early phase II trial of human rotavirus vaccine candidate RV3. *Vaccine*, 20(23-24), 2950-2956.
- Beisner, B., Kool, D., Marich, A., & Holmes, I. H. (1998). Characterisation of G serotype dependent non-antibody inhibitors of rotavirus in normal mouse serum. *Arch Virol*, 143(7), 1277-1294.
- Benhafid, M., Elomari, N., Azzouzi Idrissi, M., Rguig, A., Gentsch, J. R., Parashar, U., et al. (2015). Effect of monovalent rotavirus vaccine on rotavirus disease burden and circulating rotavirus strains among children in Morocco. *J Med Virol*, 87(6), 944-953. doi: 10.1002/jmv.24122
- Bernstein, D. I., Sack, D. A., Rothstein, E., Reisinger, K., Smith, V. E., O'Sullivan, D., et al. (1999). Efficacy of live, attenuated, human rotavirus vaccine 89-12 in infants: a randomised placebo-controlled trial. *Lancet*, 354(9175), 287-290. doi: 10.1016/S0140-6736(98)12106-2

- Bernstein, D. I., Sander, D. S., Smith, V. E., Schiff, G. M., & Ward, R. L. (1991). Protection from rotavirus reinfection: 2-year prospective study. *J Infect Dis*, *164*(2), 277-283.
- Bernstein, D. I., Smith, V. E., Sander, D. S., Pax, K. A., Schiff, G. M., & Ward, R. L. (1990). Evaluation of WC3 rotavirus vaccine and correlates of protection in healthy infants. *J Infect Dis*, *162*(5), 1055-1062.
- Berois, M., Libersou, S., Russi, J., Arbiza, J., & Cohen, J. (2003). Genetic variation in the VP7 gene of human rotavirus isolated in Montevideo-Uruguay from 1996-1999. *J Med Virol*, *71*(3), 456-462. doi: 10.1002/jmv.10511
- Binka, E., Vermund, S. H., & Armah, G. E. (2011). Rotavirus diarrhea among children less than 5 years of age in urban Ghana. *Pediatr Infect Dis J*, *30*(8), 716-718. doi: 10.1097/INF.0b013e318223bd85
- Binka, F. N., Anto, F. K., Oduro, A. R., Awini, E. A., Nazzar, A. K., Armah, G. E., et al. (2003). Incidence and risk factors of paediatric rotavirus diarrhoea in northern Ghana. *Trop Med Int Health*, *8*(9), 840-846.
- Bishop, R. F., Barnes, G. L., Cipriani, E., & Lund, J. S. (1983). Clinical immunity after neonatal rotavirus infection. A prospective longitudinal study in young children. *N Engl J Med*, *309*(2), 72-76. doi: 10.1056/NEJM198307143090203
- Blutt, S. E., Kirkwood, C. D., Parreno, V., Warfield, K. L., Ciarlet, M., Estes, M. K., et al. (2003). Rotavirus antigenaemia and viraemia: a common event? *Lancet*, *362*(9394), 1445-1449. doi: 10.1016/S0140-6736(03)14687-9
- Blutt, S. E., Matson, D. O., Crawford, S. E., Staat, M. A., Azimi, P., Bennett, B. L., et al. (2007). Rotavirus antigenemia in children is associated with viremia. *PLoS Med*, *4*(4), e121. doi: 10.1371/journal.pmed.0040121
- Boom, J. A., Sahni, L. C., Payne, D. C., Gautam, R., Lyde, F., Mijatovic-Rustempasic, S., et al. (2012). Symptomatic infection and detection of vaccine and vaccine-reassortant

- rotavirus strains in 5 children: a case series. *J Infect Dis*, 206(8), 1275-1279. doi: 10.1093/infdis/jis490
- Bremont, M., Juste-Lesage, P., Chabanne-Vautherot, D., Charpilienne, A., & Cohen, J. (1992). Sequences of the four larger proteins of a porcine group C rotavirus and comparison with the equivalent group A rotavirus proteins. *Virology*, 186(2), 684-692.
- Bucardo, F., Lindgren, P. E., Svensson, L., & Nordgren, J. (2011). Low prevalence of rotavirus and high prevalence of norovirus in hospital and community wastewater after introduction of rotavirus vaccine in Nicaragua. *PLoS One*, 6(10), e25962. doi: 10.1371/journal.pone.0025962
- Buttery, J. P., Lambert, S. B., Grimwood, K., Nissen, M. D., Field, E. J., Macartney, K. K., et al. (2011). Reduction in rotavirus-associated acute gastroenteritis following introduction of rotavirus vaccine into Australia's National Childhood vaccine schedule. *Pediatr Infect Dis J*, 30(1 Suppl), S25-29. doi: 10.1097/INF.0b013e3181fefdee
- Butz, A. M., Fosarelli, P., Dick, J., Cusack, T., & Yolken, R. (1993). Prevalence of rotavirus on high-risk fomites in day-care facilities. *Pediatrics*, 92(2), 202-205.
- Carlson, J. A., Middleton, P. J., Szymanski, M. T., Huber, J., & Petric, M. (1978). Fatal rotavirus gastroenteritis: an analysis of 21 cases. *Am J Dis Child*, 132(5), 477-479.
- Caul, E. O., Ashley, C. R., Darville, J. M., & Bridger, J. C. (1990). Group C rotavirus associated with fatal enteritis in a family outbreak. *J Med Virol*, 30(3), 201-205.
- Centers for Disease, C., & Prevention. (1999). Intussusception among recipients of rotavirus vaccine--United States, 1998-1999. *MMWR Morb Mortal Wkly Rep*, 48(27), 577-581.

- Chemello, M. E., Aristimuno, O. C., Michelangeli, F., & Ruiz, M. C. (2002). Requirement for vacuolar H⁺ -ATPase activity and Ca²⁺ gradient during entry of rotavirus into MA104 cells. *J Virol*, 76(24), 13083-13087.
- Chen, D., Luongo, C. L., Nibert, M. L., & Patton, J. T. (1999). Rotavirus open cores catalyze 5'-capping and methylation of exogenous RNA: evidence that VP3 is a methyltransferase. *Virology*, 265(1), 120-130. doi: 10.1006/viro.1999.0029
- Chiappini, E., Azzari, C., Moriondo, M., Galli, L., & de Martino, M. (2005). Viraemia is a common finding in immunocompetent children with rotavirus infection. *J Med Virol*, 76(2), 265-267. doi: 10.1002/jmv.20351
- Chiu, C., & Miller, S. (2016). Next-generation sequencing *Molecular Microbiology* (pp. 68-79): American Society of Microbiology.
- Chnaiderman, J., Barro, M., & Spencer, E. (2002). NSP5 phosphorylation regulates the fate of viral mRNA in rotavirus infected cells. *Arch Virol*, 147(10), 1899-1911. doi: 10.1007/s00705-002-0856-9
- Clark, H. F., Bernstein, D. I., Dennehy, P. H., Offit, P., Pichichero, M., Treanor, J., et al. (2004). Safety, efficacy, and immunogenicity of a live, quadrivalent human-bovine reassortant rotavirus vaccine in healthy infants. *J Pediatr*, 144(2), 184-190. doi: 10.1016/j.jpeds.2003.10.054
- Clark, H. F., Borian, F. E., Modesto, K., & Plotkin, S. A. (1990). Serotype 1 reassortant of bovine rotavirus WC3, strain WI79-9, induces a polytypic antibody response in infants. *Vaccine*, 8(4), 327-332.
- Clark, H. F., Burke, C. J., Volkin, D. B., Offit, P., Ward, R. L., Bresee, J. S., et al. (2003). Safety, immunogenicity and efficacy in healthy infants of G1 and G2 human reassortant rotavirus vaccine in a new stabilizer/buffer liquid formulation. *Pediatr Infect Dis J*, 22(10), 914-920. doi: 10.1097/01.inf.0000091887.48999.77

- Clark, H. F., & Offit, P. A. (2004). Vaccines for rotavirus gastroenteritis universally needed for infants. *Pediatr Ann*, 33(8), 536-543.
- Clark, J. D., Hill, S. M., & Phillips, A. D. (1988). Investigation of hospital-acquired rotavirus gastroenteritis using RNA electrophoresis. *J Med Virol*, 26(3), 289-299.
- Clark, K. J., Tamborello, T. J., Xu, Z., Mann, F. E., Jr., Bonnot, C. E., & Woode, G. N. (1996). An unusual group-A rotavirus associated with an epidemic of diarrhea among three-month-old calves. *J Am Vet Med Assoc*, 208(4), 552-554.
- Clements-Mann, M. L., Dudas, R., Hoshino, Y., Nehring, P., Sperber, E., Wagner, M., et al. (2001). Safety and immunogenicity of live attenuated quadrivalent human-bovine (UK) reassortant rotavirus vaccine administered with childhood vaccines to infants. *Vaccine*, 19(32), 4676-4684.
- Cohen, J., Charpilienne, A., Chilmonczyk, S., & Estes, M. K. (1989). Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. *Virology*, 171(1), 131-140.
- Cortese, M. M., Parashar, U. D., Centers for Disease, C., & Prevention. (2009). Prevention of rotavirus gastroenteritis among infants and children: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep*, 58(RR-2), 1-25.
- Crawford, S. E., Labbe, M., Cohen, J., Burroughs, M. H., Zhou, Y. J., & Estes, M. K. (1994). Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. *J Virol*, 68(9), 5945-5952.
- Curns, A. T., Panozzo, C. A., Tate, J. E., Payne, D. C., Patel, M. M., Cortese, M. M., et al. (2011). Remarkable postvaccination spatiotemporal changes in United States rotavirus activity. *Pediatr Infect Dis J*, 30(1 Suppl), S54-55. doi: 10.1097/INF.0b013e3181fefda9

- Dalgic, N., Sancar, M., Bayraktar, B., Pullu, M., & Hasim, O. (2011). Probiotic, zinc and lactose-free formula in children with rotavirus diarrhea: are they effective? *Pediatr Int*, 53(5), 677-682. doi: 10.1111/j.1442-200X.2011.03325.x
- Damanka, S., Adiku, T. K., Armah, G. E., Rodrigues, O., Donkor, E. S., Nortey, D., et al. (2016). Rotavirus Infection in Children with Diarrhea at Korle-Bu Teaching Hospital, Ghana. *Jpn J Infect Dis*, 69(4), 331-334. doi: 10.7883/yoken.JJID.2014.407
- Dennehy, P. H. (2008). Rotavirus vaccines: an overview. *Clin Microbiol Rev*, 21(1), 198-208. doi: 10.1128/CMR.00029-07
- Dennehy, P. H., Brady, R. C., Halperin, S. A., Ward, R. L., Alvey, J. C., Fischer, F. H., Jr., et al. (2005). Comparative evaluation of safety and immunogenicity of two dosages of an oral live attenuated human rotavirus vaccine. *Pediatr Infect Dis J*, 24(6), 481-488.
- Dennis, F. E., Fujii, Y., Haga, K., Damanka, S., Lartey, B., Agbemabiese, C. A., et al. (2014). Identification of novel Ghanaian G8P[6] human-bovine reassortant rotavirus strain by next generation sequencing. *PLoS One*, 9(6), e100699. doi: 10.1371/journal.pone.0100699
- Desselberger, U. (2014). Rotaviruses. *Virus Res*, 190, 75-96.
- Doll, M. K., Buckeridge, D. L., Morrison, K. T., Gagneur, A., Tapiero, B., Charest, H., et al. (2015). Effectiveness of monovalent rotavirus vaccine in a high-income, predominant-use setting. *Vaccine*, 33(51), 7307-7314. doi: 10.1016/j.vaccine.2015.10.118
- Donato, C. M., Ch'ng, L. S., Boniface, K. F., Crawford, N. W., Buttery, J. P., Lyon, M., et al. (2012). Identification of strains of RotaTeq rotavirus vaccine in infants with gastroenteritis following routine vaccination. *J Infect Dis*, 206(3), 377-383. doi: 10.1093/infdis/jis361

- Dormitzer, P. R., Greenberg, H. B., & Harrison, S. C. (2001). Proteolysis of monomeric recombinant rotavirus VP4 yields an oligomeric VP5* core. *J Virol*, *75*(16), 7339-7350. doi: 10.1128/JVI.75.16.7339-7350.2001
- Dormitzer, P. R., Sun, Z. Y., Blixt, O., Paulson, J. C., Wagner, G., & Harrison, S. C. (2002). Specificity and affinity of sialic acid binding by the rhesus rotavirus VP8* core. *J Virol*, *76*(20), 10512-10517.
- Enweronu-Laryea, C. C., Boamah, I., Sifah, E., Diamenu, S. K., & Armah, G. (2014a). Decline in severe diarrhea hospitalizations after the introduction of rotavirus vaccination in Ghana: a prevalence study. *BMC Infect Dis*, *14*, 431. doi: 10.1186/1471-2334-14-431
- Enweronu-Laryea, C. C., Sagoe, K. W., Damanka, S., Lartey, B., & Armah, G. E. (2013). Rotavirus genotypes associated with childhood severe acute diarrhoea in southern Ghana: a cross-sectional study. *Virol J*, *10*, 287. doi: 10.1186/1743-422X-10-287
- Enweronu-Laryea, C. C., Sagoe, K. W., Glover-Addy, H., Asmah, R. H., Mingle, J. A., & Armah, G. E. (2012). Prevalence of severe acute rotavirus gastroenteritis and intussusceptions in Ghanaian children under 5 years of age. *J Infect Dev Ctries*, *6*(2), 148-155.
- Enweronu-Laryea, C. C., Sagoe, K. W., Mwenda, J. M., & Armah, G. E. (2014b). Severe acute rotavirus gastroenteritis in children less than 5 years in southern Ghana: 2006-2011. *Pediatr Infect Dis J*, *33 Suppl 1*, S9-S13. doi: 10.1097/INF.0000000000000045
- Esona, M. D., & Gautam, R. (2015). Rotavirus. *Clin Lab Med*, *35*(2), 363-391. doi: 10.1016/j.cll.2015.02.012
- Esquivel, F. R., Lopez, S., Guitierrez, X. L., & Arias, C. (2000). The internal rotavirus protein VP6 primes for an enhanced neutralizing antibody response. *Arch Virol*, *145*(4), 813-825.

- Estes, M., & Greenberg, H. (2013). Rotaviruses, p 1347–1401. *Fields virology, 6th ed.* Lippincott Williams & Wilkins, Philadelphia, PA.
- Estes, M., & Kapikian, A. (2007). Rotaviruses, p 1917–1974. *Fields virology, 2.*
- Estes, M. K., Conner, M. E., Gilger, M. A., & Graham, D. Y. (1989). Molecular biology and immunology of rotavirus infections. *Immunol Invest, 18*(1-4), 571-581.
- Estes, M. K., Crawford, S. E., Penaranda, M. E., Petrie, B. L., Burns, J. W., Chan, W. K., et al. (1987). Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system. *J Virol, 61*(5), 1488-1494.
- Fischer, T. K., Ashley, D., Kerin, T., Reynolds-Hedmann, E., Gentsch, J., Widdowson, M. A., et al. (2005). Rotavirus antigenemia in patients with acute gastroenteritis. *J Infect Dis, 192*(5), 913-919. doi: 10.1086/432549
- Flores, J., Perez-Schael, I., Blanco, M., White, L., Garcia, D., Vilar, M., et al. (1990). Comparison of reactogenicity and antigenicity of M37 rotavirus vaccine and rhesus-rotavirus-based quadrivalent vaccine. *Lancet, 336*(8711), 330-334.
- Franco, M. A., Angel, J., & Greenberg, H. B. (2006). Immunity and correlates of protection for rotavirus vaccines. *Vaccine, 24*(15), 2718-2731. doi: 10.1016/j.vaccine.2005.12.048
- From the CDC: Rotavirus vaccine withdrawn from market. (1999). *J Am Osteopath Assoc, 99*(11), 566f. doi: 10.7556/jaoa.1999.99.11.566f
- Gardet, A., Breton, M., Fontanges, P., Trugnan, G., & Chwetzoff, S. (2006). Rotavirus spike protein VP4 binds to and remodels actin bundles of the epithelial brush border into actin bodies. *J Virol, 80*(8), 3947-3956. doi: 10.1128/JVI.80.8.3947-3956.2006
- Gentsch, J. R., Glass, R. I., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., et al. (1992). Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J Clin Microbiol, 30*(6), 1365-1373.

- Glass, R. I., Lang, D. R., Ivanoff, B. N., & Compans, R. W. (1996). Introduction: rotavirus--from basic research to a vaccine. *J Infect Dis*, *174 Suppl 1*, S1-2.
- Glass, R. I., & Parashar, U. D. (2014). Rotavirus vaccines--balancing intussusception risks and health benefits. *N Engl J Med*, *370*(6), 568-570. doi: 10.1056/NEJMe1315836
- Gonzalez, R. A., Espinosa, R., Romero, P., Lopez, S., & Arias, C. F. (2000). Relative localization of viroplasmic and endoplasmic reticulum-resident rotavirus proteins in infected cells. *Arch Virol*, *145*(9), 1963-1973.
- Goodwin, S., McPherson, J. D., & McCombie, W. R. (2016). Coming of age: ten years of next-generation sequencing technologies. *Nature Reviews Genetics*, *17*(6), 333-351.
- Gouvea, V., Glass, R. I., Woods, P., Taniguchi, K., Clark, H. F., Forrester, B., et al. (1990). Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J Clin Microbiol*, *28*(2), 276-282.
- Hagbom, M., Istrate, C., Engblom, D., Karlsson, T., Rodriguez-Diaz, J., Buesa, J., et al. (2011). Rotavirus stimulates release of serotonin (5-HT) from human enterochromaffin cells and activates brain structures involved in nausea and vomiting. *PLoS Pathog*, *7*(7), e1002115. doi: 10.1371/journal.ppat.1002115
- Hanquet, G., Ducoffre, G., Vergison, A., Neels, P., Sabbe, M., Van Damme, P., et al. (2011). Impact of rotavirus vaccination on laboratory confirmed cases in Belgium. *Vaccine*, *29*(29-30), 4698-4703. doi: 10.1016/j.vaccine.2011.04.098
- Hashizume, M., Armstrong, B., Wagatsuma, Y., Faruque, A. S., Hayashi, T., & Sack, D. A. (2008). Rotavirus infections and climate variability in Dhaka, Bangladesh: a time-series analysis. *Epidemiol Infect*, *136*(9), 1281-1289. doi: 10.1017/S0950268807009776

- Hsieh, Y. C., Wu, F. T., Hsiung, C. A., Wu, H. S., Chang, K. Y., & Huang, Y. C. (2014). Comparison of virus shedding after lived attenuated and pentavalent reassortant rotavirus vaccine. *Vaccine*, *32*(10), 1199-1204. doi: 10.1016/j.vaccine.2013.08.041
- Hu, L., Crawford, S. E., Hyser, J. M., Estes, M. K., & Prasad, B. V. (2012). Rotavirus non-structural proteins: structure and function. *Curr Opin Virol*, *2*(4), 380-388. doi: 10.1016/j.coviro.2012.06.003
- Hyser, J. M., Collinson-Pautz, M. R., Utama, B., & Estes, M. K. (2010). Rotavirus disrupts calcium homeostasis by NSP4 viroporin activity. *MBio*, *1*(5). doi: 10.1128/mBio.00265-10
- Hyser, J. M., Utama, B., Crawford, S. E., & Estes, M. K. (2012). Genetic divergence of rotavirus nonstructural protein 4 results in distinct serogroup-specific viroporin activity and intracellular punctate structure morphologies. *J Virol*, *86*(9), 4921-4934. doi: 10.1128/JVI.06759-11
- Iturriza-Gomara, M., Dallman, T., Banyai, K., Bottiger, B., Buesa, J., Diedrich, S., et al. (2011). Rotavirus genotypes co-circulating in Europe between 2006 and 2009 as determined by EuroRotaNet, a pan-European collaborative strain surveillance network. *Epidemiol Infect*, *139*(6), 895-909. doi: 10.1017/S0950268810001810
- Jayaram, H., Taraporewala, Z., Patton, J. T., & Prasad, B. V. (2002). Rotavirus protein involved in genome replication and packaging exhibits a HIT-like fold. *Nature*, *417*(6886), 311-315. doi: 10.1038/417311a
- Jiang, X., Jayaram, H., Kumar, M., Ludtke, S. J., Estes, M. K., & Prasad, B. V. (2006). Cryoelectron microscopy structures of rotavirus NSP2-NSP5 and NSP2-RNA complexes: implications for genome replication. *J Virol*, *80*(21), 10829-10835. doi: 10.1128/JVI.01347-06

- Jourdan, N., Maurice, M., Delautier, D., Quero, A. M., Servin, A. L., & Trugnan, G. (1997). Rotavirus is released from the apical surface of cultured human intestinal cells through nonconventional vesicular transport that bypasses the Golgi apparatus. *J Virol*, *71*(11), 8268-8278.
- Kaila, M., Isolauri, E., Saxelin, M., Arvilommi, H., & Vesikari, T. (1995). Viable versus inactivated lactobacillus strain GG in acute rotavirus diarrhoea. *Arch Dis Child*, *72*(1), 51-53.
- Kattoura, M. D., Chen, X., & Patton, J. T. (1994). The rotavirus RNA-binding protein NS35 (NSP2) forms 10S multimers and interacts with the viral RNA polymerase. *Virology*, *202*(2), 803-813. doi: 10.1006/viro.1994.1402
- Kirkwood, C. D., Ma, L. F., Carey, M. E., & Steele, A. D. (2017). The rotavirus vaccine development pipeline. *Vaccine*. doi: 10.1016/j.vaccine.2017.03.076
- Koopmans, M., & Van Asperen, I. (1999). Epidemiology of rotavirus infections in The Netherlands. *Acta Paediatr Suppl*, *88*(426), 31-37.
- Kovacs-Nolan, J., Yoo, D., & Mine, Y. (2003). Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus. *Biochem J*, *376*(Pt 1), 269-275. doi: 10.1042/BJ20021969
- Larralde, G., Li, B. G., Kapikian, A. Z., & Gorziglia, M. (1991). Serotype-specific epitope(s) present on the VP8 subunit of rotavirus VP4 protein. *J Virol*, *65*(6), 3213-3218.
- Lawton, J. A., Estes, M. K., & Prasad, B. V. (2001). Identification and characterization of a transcription pause site in rotavirus. *J Virol*, *75*(4), 1632-1642. doi: 10.1128/JVI.75.4.1632-1642.2001
- Li, N., & Wang, Z. Y. (2003). Viremia and extraintestinal infections in infants with rotavirus diarrhea. *Di Yi Jun Yi Da Xue Xue Bao*, *23*(7), 643-648.

- Linhares, A. C., & Justino, M. C. (2014). Rotavirus vaccination in Brazil: effectiveness and health impact seven years post-introduction. *Expert Rev Vaccines*, *13*(1), 43-57. doi: 10.1586/14760584.2014.861746
- Liu, N., Yen, C., Fang, Z. Y., Tate, J. E., Jiang, B., Parashar, U. D., et al. (2012). Projected health impact and cost-effectiveness of rotavirus vaccination among children <5 years of age in China. *Vaccine*, *30*(48), 6940-6945. doi: 10.1016/j.vaccine.2012.05.084
- Lu, X., McDonald, S. M., Tortorici, M. A., Tao, Y. J., Vasquez-Del Carpio, R., Nibert, M. L., et al. (2008). Mechanism for coordinated RNA packaging and genome replication by rotavirus polymerase VP1. *Structure*, *16*(11), 1678-1688. doi: 10.1016/j.str.2008.09.006
- Luchs, A., Morillo, S. G., Ribeiro, C. D., Cilli, A., Calux, S. J., Carmona Rde, C., et al. (2013). Rotavirus G2P[4] and G2P[4]+[6] infections during norovirus gastroenteritis outbreak: summer season 2010, Brazil. *Rev Soc Bras Med Trop*, *46*(2), 227-230. doi: 10.1590/0037-8682-1162-2013
- Ludert, J. E., Gil, F., Liprandi, F., & Esparza, J. (1986). The structure of the rotavirus inner capsid studied by electron microscopy of chemically disrupted particles. *J Gen Virol*, *67* (Pt 8), 1721-1725. doi: 10.1099/0022-1317-67-8-1721
- Ludert, J. E., Ruiz, M. C., Hidalgo, C., & Liprandi, F. (2002). Antibodies to rotavirus outer capsid glycoprotein VP7 neutralize infectivity by inhibiting virion decapsidation. *J Virol*, *76*(13), 6643-6651.
- Lundgren, O., Peregrin, A. T., Persson, K., Kordasti, S., Uhnoo, I., & Svensson, L. (2000). Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhea. *Science*, *287*(5452), 491-495.
- Lundgren, O., & Svensson, L. (2001). Pathogenesis of rotavirus diarrhea. *Microbes Infect*, *3*(13), 1145-1156.

- Lynch, M., Shieh, W. J., Tatti, K., Gentsch, J. R., Ferebee-Harris, T., Jiang, B., et al. (2003). The pathology of rotavirus-associated deaths, using new molecular diagnostics. *Clin Infect Dis*, 37(10), 1327-1333. doi: 10.1086/379322
- Majamaa, H., Isolauri, E., Saxelin, M., & Vesikari, T. (1995). Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. *J Pediatr Gastroenterol Nutr*, 20(3), 333-338.
- Malik, J., Bhan, M. K., & Ray, P. (2008). Natural immunity to rotavirus infection in children. *Indian J Biochem Biophys*, 45(4), 219-228.
- Mansell, E. A., & Patton, J. T. (1990). Rotavirus RNA replication: VP2, but not VP6, is necessary for viral replicase activity. *J Virol*, 64(10), 4988-4996.
- Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S. M., et al. (2008). Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. *J Virol*, 82(7), 3204-3219. doi: 10.1128/JVI.02257-07
- Matthijnssens, J., Ciarlet, M., McDonald, S. M., Attoui, H., Banyai, K., Brister, J. R., et al. (2011). Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). *Arch Virol*, 156(8), 1397-1413. doi: 10.1007/s00705-011-1006-z
- Matthijnssens, J., Mino, S., Papp, H., Potgieter, C., Novo, L., Heylen, E., et al. (2012a). Complete molecular genome analyses of equine rotavirus A strains from different continents reveal several novel genotypes and a largely conserved genotype constellation. *J Gen Virol*, 93(Pt 4), 866-875. doi: 10.1099/vir.0.039255-0
- Matthijnssens, J., Nakagomi, O., Kirkwood, C. D., Ciarlet, M., Desselberger, U., & Van Ranst, M. (2012b). Group A rotavirus universal mass vaccination: how and to what

- extent will selective pressure influence prevalence of rotavirus genotypes? *Expert Rev Vaccines*, *11*(11), 1347-1354. doi: 10.1586/erv.12.105
- Matthijnssens, J., Otto, P. H., Ciarlet, M., Desselberger, U., Van Ranst, M., & Johne, R. (2012c). VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. *Arch Virol*, *157*(6), 1177-1182. doi: 10.1007/s00705-012-1273-3
- McClain, B., Settembre, E., Temple, B. R., Bellamy, A. R., & Harrison, S. C. (2010). X-ray crystal structure of the rotavirus inner capsid particle at 3.8 Å resolution. *J Mol Biol*, *397*(2), 587-599. doi: 10.1016/j.jmb.2010.01.055
- McDonald, S. M., & Patton, J. T. (2008). Molecular characterization of a subgroup specificity associated with the rotavirus inner capsid protein VP2. *J Virol*, *82*(6), 2752-2764. doi: 10.1128/JVI.02492-07
- McDonald, S. M., & Patton, J. T. (2011). Assortment and packaging of the segmented rotavirus genome. *Trends Microbiol*, *19*(3), 136-144. doi: 10.1016/j.tim.2010.12.002
- Meeting of the Strategic Advisory Group of Experts on immunization, October 2009 - conclusions and recommendations. (2009). *Wkly Epidemiol Rec*, *84*(50), 517-532.
- Midthun, K., Halsey, N. A., Jett-Goheen, M., Clements, M. L., Steinhoff, M., King, J. C., et al. (1991). Safety and immunogenicity of human rotavirus vaccine strain M37 in adults, children, and infants. *J Infect Dis*, *164*(4), 792-796.
- Moon, S., Wang, Y., Dennehy, P., Simonsen, K. A., Zhang, J., & Jiang, B. (2012). Antigenemia, RNAemia, and innate immunity in children with acute rotavirus diarrhea. *FEMS Immunol Med Microbiol*, *64*(3), 382-391. doi: 10.1111/j.1574-695X.2011.00923.x
- Morris, A. P., & Estes, M. K. (2001). Microbes and microbial toxins: paradigms for microbial-mucosal interactions. VIII. Pathological consequences of rotavirus infection and its enterotoxin. *Am J Physiol Gastrointest Liver Physiol*, *281*(2), G303-310.

- Morrison, S. C., & Czinn, S. J. (2001). Portal vein gas associated with rotavirus infection. *J Pediatr Gastroenterol Nutr*, 33(5), 626-628.
- Mpabalwani, E. M., Simwaka, C. J., Mwenda, J. M., Mubanga, C. P., Monze, M., Matapo, B., et al. (2016). Impact of Rotavirus Vaccination on Diarrheal Hospitalizations in Children Aged <5 Years in Lusaka, Zambia. *Clin Infect Dis*, 62 Suppl 2, S183-187. doi: 10.1093/cid/civ1027
- Mukhopadhyaya, I., Murdoch, H., Berry, S., Hunt, A., Iturriza-Gomara, M., Smith-Palmer, A., et al. (2017). Changing molecular epidemiology of rotavirus infection after introduction of monovalent rotavirus vaccination in Scotland. *Vaccine*, 35(1), 156-163. doi: 10.1016/j.vaccine.2016.11.028
- Musalem, C., & Espejo, R. T. (1985). Release of progeny virus from cells infected with simian rotavirus SA11. *J Gen Virol*, 66 (Pt 12), 2715-2724. doi: 10.1099/0022-1317-66-12-2715
- Mwenda, J. M., Ntoto, K. M., Abebe, A., Enweronu-Laryea, C., Amina, I., McHomvu, J., et al. (2010). Burden and epidemiology of rotavirus diarrhea in selected African countries: preliminary results from the African Rotavirus Surveillance Network. *J Infect Dis*, 202 Suppl, S5-S11. doi: 10.1086/653557
- Nakagomi, T., & Nakagomi, O. (2005). Rotavirus antigenemia in children with encephalopathy accompanied by rotavirus gastroenteritis. *Arch Virol*, 150(9), 1927-1931. doi: 10.1007/s00705-005-0565-2
- Nakano, I., Taniguchi, K., Ishibashi-Ueda, H., Maeno, Y., Yamamoto, N., Yui, A., et al. (2011). Sudden death from systemic rotavirus infection and detection of nonstructural rotavirus proteins. *J Clin Microbiol*, 49(12), 4382-4385. doi: 10.1128/JCM.01303-11
- Nyaga, M. M., Jere, K. C., Esona, M. D., Seheri, M. L., Stucker, K. M., Halpin, R. A., et al. (2015). Whole genome detection of rotavirus mixed infections in human, porcine and

- bovine samples co-infected with various rotavirus strains collected from sub-Saharan Africa. *Infect Genet Evol*, 31, 321-334. doi: 10.1016/j.meegid.2015.02.011
- Pant, N., Marcotte, H., Brussow, H., Svensson, L., & Hammarstrom, L. (2007). Effective prophylaxis against rotavirus diarrhea using a combination of *Lactobacillus rhamnosus* GG and antibodies. *BMC Microbiol*, 7, 86. doi: 10.1186/1471-2180-7-86
- Parashar, U. D., Alexander, J. P., Glass, R. I., Advisory Committee on Immunization Practices, C. f. D. C., & Prevention. (2006a). Prevention of rotavirus gastroenteritis among infants and children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep*, 55(RR-12), 1-13.
- Parashar, U. D., Bresee, J. S., Gentsch, J. R., & Glass, R. I. (1998). Rotavirus. *Emerg Infect Dis*, 4(4), 561-570. doi: 10.3201/eid0404.980406
- Parashar, U. D., Gibson, C. J., Bresee, J. S., & Glass, R. I. (2006b). Rotavirus and severe childhood diarrhea. *Emerg Infect Dis*, 12(2), 304-306. doi: 10.3201/eid1202.050006
- Parashar, U. D., & Glass, R. I. (2006). Public health. Progress toward rotavirus vaccines. *Science*, 312(5775), 851-852. doi: 10.1126/science.1128827
- Patel, M. M., Pitzer, V. E., Alonso, W. J., Vera, D., Lopman, B., Tate, J., et al. (2013). Global seasonality of rotavirus disease. *Pediatr Infect Dis J*, 32(4), e134-147. doi: 10.1097/INF.0b013e31827d3b68
- Patton, J. T., Jones, M. T., Kalbach, A. N., He, Y. W., & Xiaobo, J. (1997). Rotavirus RNA polymerase requires the core shell protein to synthesize the double-stranded RNA genome. *J Virol*, 71(12), 9618-9626.
- Patton, J. T., Silvestri, L. S., Tortorici, M. A., Vasquez-Del Carpio, R., & Taraporewala, Z. F. (2006). Rotavirus genome replication and morphogenesis: role of the viroplasm. *Curr Top Microbiol Immunol*, 309, 169-187.

- Patton, J. T., Wentz, M., Xiaobo, J., & Ramig, R. F. (1996). cis-Acting signals that promote genome replication in rotavirus mRNA. *J Virol*, *70*(6), 3961-3971.
- Paulke-Korinek, M., Rendi-Wagner, P., Kundi, M., Kronik, R., & Kollaritsch, H. (2010). Universal mass vaccination against rotavirus gastroenteritis: impact on hospitalization rates in austrian children. *Pediatr Infect Dis J*, *29*(4), 319-323. doi: 10.1097/INF.0b013e3181c18434
- Payne, D. C., Boom, J. A., Staat, M. A., Edwards, K. M., Szilagyi, P. G., Klein, E. J., et al. (2013). Effectiveness of pentavalent and monovalent rotavirus vaccines in concurrent use among US children <5 years of age, 2009-2011. *Clin Infect Dis*, *57*(1), 13-20. doi: 10.1093/cid/cit164
- Payne, D. C., Edwards, K. M., Bowen, M. D., Keckley, E., Peters, J., Esona, M. D., et al. (2010). Sibling transmission of vaccine-derived rotavirus (RotaTeq) associated with rotavirus gastroenteritis. *Pediatrics*, *125*(2), e438-441. doi: 10.1542/peds.2009-1901
- Phua, K. B., Quak, S. H., Lee, B. W., Emmanuel, S. C., Goh, P., Han, H. H., et al. (2005). Evaluation of RIX4414, a live, attenuated rotavirus vaccine, in a randomized, double-blind, placebo-controlled phase 2 trial involving 2464 Singaporean infants. *J Infect Dis*, *192 Suppl 1*, S6-S16. doi: 10.1086/431511
- Poncet, D., Aponte, C., & Cohen, J. (1993). Rotavirus protein NSP3 (NS34) is bound to the 3' end consensus sequence of viral mRNAs in infected cells. *J Virol*, *67*(6), 3159-3165.
- Raes, M., Strens, D., Vergison, A., Verghote, M., & Standaert, B. (2011). Reduction in pediatric rotavirus-related hospitalizations after universal rotavirus vaccination in Belgium. *Pediatr Infect Dis J*, *30*(7), e120-125. doi: 10.1097/INF.0b013e318214b811
- Rainsford, E. W., & McCrae, M. A. (2007). Characterization of the NSP6 protein product of rotavirus gene 11. *Virus Res*, *130*(1-2), 193-201. doi: 10.1016/j.virusres.2007.06.011

- Rasanen, S., Lappalainen, S., Halkosalo, A., Salminen, M., & Vesikari, T. (2011). Rotavirus gastroenteritis in Finnish children in 2006-2008, at the introduction of rotavirus vaccination. *Scand J Infect Dis*, 43(1), 58-63. doi: 10.3109/00365548.2010.508462
- Ribas Mde, L., Castano, Y., Martinez, M. D., Tejero, Y., & Cordero, Y. (2015a). Norovirus and Rotavirus infection in children aged less than five years in a paediatric hospital, Havana, Cuba. *Braz J Infect Dis*, 19(2), 222-223. doi: 10.1016/j.bjid.2014.10.002
- Ribas Mde, L., Tejero, Y., Cordero, Y., de Los Angeles Leon, M., Rodriguez, M., Perez-Lastre, J., et al. (2015b). Detection of rotavirus and other enteropathogens in children hospitalized with acute gastroenteritis in Havana, Cuba. *Arch Virol*, 160(8), 1923-1930. doi: 10.1007/s00705-015-2458-3
- Rivera, L., Pena, L. M., Stainier, I., Gillard, P., Cheuvart, B., Smolenov, I., et al. (2011). Horizontal transmission of a human rotavirus vaccine strain--a randomized, placebo-controlled study in twins. *Vaccine*, 29(51), 9508-9513. doi: 10.1016/j.vaccine.2011.10.015
- Rotavirus vaccines WHO position paper: January 2013 - Recommendations. (2013). *Vaccine*, 31(52), 6170-6171. doi: 10.1016/j.vaccine.2013.05.037
- Rudd, C., Mwenda, J., & Chilengi, R. (2015). Rotavirus landscape in Africa-Towards prevention and control: A report of the 8th African rotavirus symposium, Livingstone, Zambia. *Vaccine*, 33(29), 3263-3267. doi: 10.1016/j.vaccine.2015.04.002
- Ruggeri, F. M., & Greenberg, H. B. (1991). Antibodies to the trypsin cleavage peptide VP8 neutralize rotavirus by inhibiting binding of virions to target cells in culture. *J Virol*, 65(5), 2211-2219.
- Ruiz-Palacios, G. M., Guerrero, M. L., Bautista-Marquez, A., Ortega-Gallegos, H., Tuz-Dzib, F., Reyes-Gonzalez, L., et al. (2007). Dose response and efficacy of a live, attenuated

- human rotavirus vaccine in Mexican infants. *Pediatrics*, 120(2), e253-261. doi: 10.1542/peds.2006-2630
- Ruiz-Palacios, G. M., Perez-Schael, I., Velazquez, F. R., Abate, H., Breuer, T., Clemens, S. C., et al. (2006). Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *N Engl J Med*, 354(1), 11-22. doi: 10.1056/NEJMoa052434
- Safadi, M. A., Berezin, E. N., Munford, V., Almeida, F. J., de Moraes, J. C., Pinheiro, C. F., et al. (2010). Hospital-based surveillance to evaluate the impact of rotavirus vaccination in Sao Paulo, Brazil. *Pediatr Infect Dis J*, 29(11), 1019-1022. doi: 10.1097/INF.0b013e3181e7886a
- Settembre, E. C., Chen, J. Z., Dormitzer, P. R., Grigorieff, N., & Harrison, S. C. (2011). Atomic model of an infectious rotavirus particle. *EMBO J*, 30(2), 408-416. doi: 10.1038/emboj.2010.322
- Silvestri, L. S., Taraporewala, Z. F., & Patton, J. T. (2004). Rotavirus replication: plus-sense templates for double-stranded RNA synthesis are made in viroplasms. *J Virol*, 78(14), 7763-7774. doi: 10.1128/JVI.78.14.7763-7774.2004
- Silvestri, L. S., Tortorici, M. A., Vasquez-Del Carpio, R., & Patton, J. T. (2005). Rotavirus glycoprotein NSP4 is a modulator of viral transcription in the infected cell. *J Virol*, 79(24), 15165-15174. doi: 10.1128/JVI.79.24.15165-15174.2005
- Stirzaker, S. C., & Both, G. W. (1989). The signal peptide of the rotavirus glycoprotein VP7 is essential for its retention in the ER as an integral membrane protein. *Cell*, 56(5), 741-747.
- Tang, B., Gilbert, J. M., Matsui, S. M., & Greenberg, H. B. (1997). Comparison of the rotavirus gene 6 from different species by sequence analysis and localization of subgroup-specific epitopes using site-directed mutagenesis. *Virology*, 237(1), 89-96. doi: 10.1006/viro.1997.8762

- Tate, J. E., Burton, A. H., Boschi-Pinto, C., Parashar, U. D., & World Health Organization-Coordinated Global Rotavirus Surveillance, N. (2016). Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013. *Clin Infect Dis*, *62 Suppl 2*, S96-S105. doi: 10.1093/cid/civ1013
- Tate, J. E., Mijatovic-Rustempasic, S., Tam, K. I., Lyde, F. C., Payne, D. C., Szilagyi, P., et al. (2013). Comparison of 2 assays for diagnosing rotavirus and evaluating vaccine effectiveness in children with gastroenteritis. *Emerg Infect Dis*, *19*(8), 1245-1252. doi: 10.3201/eid1908.130461
- Tate, J. E., Simonsen, L., Viboud, C., Steiner, C., Patel, M. M., Curns, A. T., et al. (2008). Trends in intussusception hospitalizations among US infants, 1993-2004: implications for monitoring the safety of the new rotavirus vaccination program. *Pediatrics*, *121*(5), e1125-1132. doi: 10.1542/peds.2007-1590
- Thomas, S. L., Walker, J. L., Fenty, J., Atkins, K. E., Elliot, A. J., Hughes, H. E., et al. (2017). Impact of the national rotavirus vaccination programme on acute gastroenteritis in England and associated costs averted. *Vaccine*, *35*(4), 680-686. doi: 10.1016/j.vaccine.2016.11.057
- Thongprachum, A., Khamrin, P., Saekhow, P., Pantip, C., Peerakome, S., Ushijima, H., et al. (2009). Analysis of the VP6 gene of human and porcine group A rotavirus strains with unusual subgroup specificities. *J Med Virol*, *81*(1), 183-191. doi: 10.1002/jmv.21345
- Trask, S. D., & Dormitzer, P. R. (2006). Assembly of highly infectious rotavirus particles recoated with recombinant outer capsid proteins. *J Virol*, *80*(22), 11293-11304. doi: 10.1128/JVI.01346-06

- Trojnar, E., Otto, P., Roth, B., Reetz, J., & Johne, R. (2010). The genome segments of a group D rotavirus possess group A-like conserved termini but encode group-specific proteins. *J Virol*, *84*(19), 10254-10265. doi: 10.1128/JVI.00332-10
- Turcios, R. M., Curns, A. T., Holman, R. C., Pandya-Smith, I., LaMonte, A., Bresee, J. S., et al. (2006). Temporal and geographic trends of rotavirus activity in the United States, 1997-2004. *Pediatr Infect Dis J*, *25*(5), 451-454. doi: 10.1097/01.inf.0000214987.67522.78
- Tzipori, S. R., Makin, T. J., & Smith, M. L. (1980). The clinical response of gnotobiotic calves, pigs and lambs to inoculation with human, calf, pig and foal rotavirus isolates. *Aust J Exp Biol Med Sci*, *58*(3), 309-318.
- UNICEF. (2016). Levels and trends in child mortality: Report 2015. New York: UNICEF; 2015.
- Velazquez, F. R., Matson, D. O., Calva, J. J., Guerrero, L., Morrow, A. L., Carter-Campbell, S., et al. (1996). Rotavirus infection in infants as protection against subsequent infections. *N Engl J Med*, *335*(14), 1022-1028. doi: 10.1056/NEJM199610033351404
- Vende, P., Tortorici, M. A., Taraporewala, Z. F., & Patton, J. T. (2003). Rotavirus NSP2 interferes with the core lattice protein VP2 in initiation of minus-strand synthesis. *Virology*, *313*(1), 261-273.
- Vesikari, T., Clark, H. F., Offit, P. A., Dallas, M. J., DiStefano, D. J., Goveia, M. G., et al. (2006a). Effects of the potency and composition of the multivalent human-bovine (WC3) reassortant rotavirus vaccine on efficacy, safety and immunogenicity in healthy infants. *Vaccine*, *24*(22), 4821-4829. doi: 10.1016/j.vaccine.2006.03.025
- Vesikari, T., Giaquinto, C., & Huppertz, H. I. (2006b). Clinical trials of rotavirus vaccines in Europe. *Pediatr Infect Dis J*, *25*(1 Suppl), S42-47.

- Vesikari, T., Isolauri, E., Ruuska, T., & Rautanen, T. (1987). Clinical trials of rotavirus vaccines. *Ciba Found Symp*, 128, 218-237.
- Vesikari, T., Karvonen, A., Puustinen, L., Zeng, S. Q., Szakal, E. D., Delem, A., et al. (2004). Efficacy of RIX4414 live attenuated human rotavirus vaccine in Finnish infants. *Pediatr Infect Dis J*, 23(10), 937-943.
- Vesikari, T., Karvonen, A. V., Majuri, J., Zeng, S. Q., Pang, X. L., Kohberger, R., et al. (2006c). Safety, efficacy, and immunogenicity of 2 doses of bovine-human (UK) and rhesus-rhesus-human rotavirus reassortant tetravalent vaccines in Finnish children. *J Infect Dis*, 194(3), 370-376. doi: 10.1086/505151
- Vesikari, T., Matson, D. O., Dennehy, P., Van Damme, P., Santosham, M., Rodriguez, Z., et al. (2006d). Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *N Engl J Med*, 354(1), 23-33. doi: 10.1056/NEJMoa052664
- Vesikari, T., Varis, T., Green, K., Flores, J., & Kapikian, A. Z. (1991). Immunogenicity and safety of rhesus-human rotavirus reassortant vaccines with serotype 1 or 2 VP7 specificity. *Vaccine*, 9(5), 334-339.
- Wang, C. M., Chen, S. C., & Chen, K. T. (2015). Current status of rotavirus vaccines. *World J Pediatr*, 11(4), 300-308. doi: 10.1007/s12519-015-0038-y
- Wang, Y. H., Pang, B. B., Zhou, X., Ghosh, S., Tang, W. F., Peng, J. S., et al. (2013). Complex evolutionary patterns of two rare human G3P[9] rotavirus strains possessing a feline/canine-like H6 genotype on an AU-1-like genotype constellation. *Infect Genet Evol*, 16, 103-112. doi: 10.1016/j.meegid.2013.01.016
- Ward, R. L., Sander, D. S., Schiff, G. M., & Bernstein, D. I. (1990). Effect of vaccination on serotype-specific antibody responses in infants administered WC3 bovine rotavirus before or after a natural rotavirus infection. *J Infect Dis*, 162(6), 1298-1303.

- WHO. (2009). Meeting of the Strategic Advisory Group of Experts on immunization, october 2009: Conclusions and recommendations. *Weekly Epidemiological Record*, 84(50).
- Wolf, M., Vo, P. T., & Greenberg, H. B. (2011). Rhesus rotavirus entry into a polarized epithelium is endocytosis dependent and involves sequential VP4 conformational changes. *J Virol*, 85(6), 2492-2503. doi: 10.1128/JVI.02082-10
- Yang, J., Wang, T., Wang, Y., Lu, B., Bai, X., Zhang, L., et al. (2007). Emergence of human rotavirus group a genotype G9 strains, Wuhan, China. *Emerg Infect Dis*, 13(10), 1587-1589. doi: 10.3201/eid1310.070142
- Yen, C., Jakob, K., Esona, M. D., Peckham, X., Rausch, J., Hull, J. J., et al. (2011a). Detection of fecal shedding of rotavirus vaccine in infants following their first dose of pentavalent rotavirus vaccine. *Vaccine*, 29(24), 4151-4155. doi: 10.1016/j.vaccine.2011.03.074
- Yen, C., Tate, J. E., Wenk, J. D., Harris, J. M., 2nd, & Parashar, U. D. (2011b). Diarrhea-associated hospitalizations among US children over 2 rotavirus seasons after vaccine introduction. *Pediatrics*, 127(1), e9-e15. doi: 10.1542/peds.2010-1393
- Zanardi, L. R., Haber, P., Mootrey, G. T., Niu, M. T., & Wharton, M. (2001). Intussusception among recipients of rotavirus vaccine: reports to the vaccine adverse event reporting system. *Pediatrics*, 107(6), E97.
- Zeng, S. Q., Halkosalo, A., Salminen, M., Szakal, E. D., Puustinen, L., & Vesikari, T. (2008). One-step quantitative RT-PCR for the detection of rotavirus in acute gastroenteritis. *J Virol Methods*, 153(2), 238-240. doi: 10.1016/j.jviromet.2008.08.004

APPENDIX**Preparation of Polyacrylamide Gels**

1) . 10% Resolving Gel

Table A1: Reagents for the preparation of 10% Resolving Gel

Reagents	1.5 mm Gel		0.75 mm Gel	
	1x	2x	1x	2x
dH₂O	15.8 ml	31.6 ml	9.9 ml	19.8 ml
30% Acrylamide stock	10.0 ml	20.0 ml	6.3 ml	12.5 ml
Resolving Buffer (pH 8.9)	3.75 ml	7.5 ml	2.4 ml	4.8 ml
TEMED	15 μ l	30 μ l	10 μ l	19 μ l
10% Ammonium persulphate	450 μ l	900 μ l	282 μ l	564 μ l

2) 3% Stacking Gel

Table A2: Reagents for the preparation of 3% Stacking Gel

Reagents	1.5 mm Gel		0.75 mm Gel	
	1x	2x	1x	2x
dH₂O	6.8 ml	13.6 ml	5.1 ml	10.2 ml
30% Acrylamide stock	1.6 ml	3.2 ml	1.2 ml	2.4 ml
Spacer Buffer (pH 6.7)	1.25 ml	2.5 ml	0.9 ml	1.9 ml
TEMED	5 μ l	10 μ l	4 μ l	8 μ l
10% Ammonium persulphate	150 μ l	300 μ l	112 μ l	225 μ l

Preparation of Reagents for PAGE

a. 30% Acrylamide stock

Acrylamide	30 g
N, N' –methylene bis-acrylamide	0.8 g

b. 1 N Hydrochloric acid (HCl)

Add 86 ml concentrated HCl to 910 ml of distilled water.

c. Resolving Buffer (pH 8.9)

Tris	36.3 g
1N HCl	48 ml

Dissolve and adjust the pH of 8.9 with HCl or 3M sodium hydroxide (NaOH). Make up to 100 ml with distilled water.

d. Stacking gel Buffer (pH 6.7)

Tris	5.98 g
Distilled water	50 ml

Adjust the pH to 6.7 with 1 N HCl. Make up to 100 ml with distilled water.

e. 10% (w/v) Ammonium persulphate (APS)

Dissolve 0.1 g ammonium persulphate in 1 ml of distilled water just prior to use.

f. 5x Tris-Glycine running buffer

25 mM Tris	15.1 g
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250 mM glycine 94 g

Dissolve and make up to 1000 ml with distilled water.

g. 1x Tris-glycine buffer

Dilute 200 ml 5x Tris-glycine buffer with 800 ml of distilled water. Use to run page gels.

Preparing reagents for Silver staining

Table A3: Reagents for the silver staining

REAGENTS	COMPONENTS	QUANTITY	
		ONE GEL	TWO GELS
FIXING SOLUTION 1 (40% ethanol, 5% acetic acid)	Ethanol	80 ml	160 ml
	dH ₂ O	110 ml	220 ml
	Acetic Acid	10 ml	20 ml
FIXING SOLUTION 2 (10% ethanol, 0.5% acetic acid)	Ethanol	20 ml	40 ml
	dH ₂ O	179 ml	358 ml
	Acetic Acid	1 ml	2 ml
SILVER NITRATE (AgNO ₃) STAINING SOLUTION (11mM)	AgNO ₃	0.37 g	0.74 g
	dH ₂ O	200 ml	400 ml
DEVELOPING SOLUTION	NaOH	7.5 g	15.0 g
	dH ₂ O	248 ml	496 ml
	36% formaldehyde	2 ml	4 ml
STOPPING SOLUTION (5% acetic acid)	Acetic Acid	10 ml	20 ml
	dH ₂ O		

Preparation of reagents for Extraction

a. Phenol: chloroform (1:1)

Add 1 part of phenol to 1 part of chloroform and mix well. Place solution in a dark or foil-covered bottle as it is light-sensitive

b. 1% sodium dodecyl sulphate (SDS)

10% sodium dodecyl sulphate (SDS) stock

Add 10 g of SDS to 100 ml of distilled water. Dissolve in 65°C water bath

c. 1M NaOAc with 1% sodium dodecyl sulphate (SDS), Ph 5.0

Dissolve 8.2g sodium acetate in 60 ml distilled water. Add 1 ml of 10% SDS stock and mix. Adjust pH to 5.0 with glacial acetic acid and make up to 100 ml with distilled water.

d. 6M Guanidine Isothiocyanate (GITC)

Add 7.09 g of GITC to 4 ml of distilled water to make up to 10 ml. Heat at 56°C in a water bath to dissolve the GITC crystals.

NB: Prepare solution just prior to use. Do not inhale!!

Preparation of reagents for Agarose gel electrophoresis of RT-PCR products

1. 20 x Tris-Acetic acid-EDTA buffer (TAE) pH 7.9

0.20 M Tris base	48.44 g
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0.05 M NaCl	6.81 g
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0.01 M EDTA	3.72 g
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Dissolve in distilled water and adjust pH to 7.9 with glacial acetic acid. Make up to 1000 ml with distilled water.

2. 1 X TAE (pH 7.9)

To make 1000 ml, add 50 ml of 20X TAE to 950 ml of distilled water.

3. 1.5% Agarose gel

Mix 1.50 g agarose in 100 ml 1X TAE (pH 7.9)

Heat in the microwave until the agarose is completely dissolved. Make solution up to 100 ml with distilled water.

After cooling, add 5.0 μ l Ethidium Bromide stock solution and pour gel into cassette tray. Insert comb and allow gel to set for at least 30 minutes before use.

Library Preparation for Whole Genome Sequencing**Table A4: RNA Fragmentation and Priming**

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Purified dsRNA (10-100) ng	5 μ l	
NEBNext First Strand Synthesis Reaction buffer (5X)	4 μ l	104 μ l
Random primers	1 μ l	26
Total volume	10 μ l	5

Table A5: Master Mix for first strand synthesis

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Murine RNase inhibitor	0.5 μ l	13 μ l
Protoscript II Reverse Transcriptase	1 μ l	26 μ l
Nuclease free water	8.5 μ l	221 μ l
Total volume	10 μ l	Total volume = 260 μ l

Table A6: Second strand cDNA synthesis

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Second round synthesis buffer	8 μ l	208 μ l
Second strand synthesis enzyme mix	4 μ l	104 μ l
Nuclease free water	48 μ l	1248 μ l
Total volume	60 μ l	Total volume = 1560 μ l

Table A7: Master Mix for End Repair/dA-tail of cDNA Library

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Second round synthesis buffer	8 μ l	208 μ l
Second strand synthesis enzyme mix	4 μ l	104 μ l
Nuclease free water	48 μ l	1248 μ l
Total volume	60 μ l	Total volume = 1560 μ l

Table A8: Master Mix for Adapter ligation

Reagents	Volume/reaction	N=24 reactions + 2 (26)
NEBNext End Reaction buffer (10X)	6.5 μ l	169 μ l
NEB End Prep Enzyme Mix	3 μ l	78 μ l
Total volume	9.5 μ l	Total volume = 247 μ l
Reagents	Volume/reaction	N=24 reactions + 2 (26)
NEBNext Q5 Hot Start High PCR Master mix	25 μ l	650 μ l
Universal PCR Primer	2.5 μ l	65 μ l
Total volume	27.5 μ l	Total volume = 715 μ l

Table A9: Master Mix for PCR Library Enrichment

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Blunt/TA Ligase Master Mix	15 μ l	390 μ l
Nuclease free water	2.5 μ l	65 μ l
Total volume	17.5 μ l	Total volume = 455 μ l

Table A10: Normalization of Qubit Standard

Lab ID	Conc. (ng/ml) Qubit	Actual Conc. (ng/ul)	Prepare 0.2 ng/ul, pipet	NF H2O need (ul)		Normalized concentration
1	79.3	7.93	2.52	97.48	100.00	0.2
2	24.2	2.42	8.26	91.74	100.00	0.2
3	3.53	0.353	5.67	4.33	10.00	0.2
4	46.4	4.64	4.31	95.69	100.00	0.2
5	1.7	0.17	11.76	-1.76	10.00	0.2
6	19.6	1.96	10.20	89.80	100.00	0.2
7	67.9	6.79	2.95	97.05	100.00	0.2
8	26.1	2.61	7.66	92.34	100.00	0.2
9	286	28.6	6.99	993.01	1000.00	0.2
10	4.46	0.446	4.48	5.52	10.00	0.2
11	253	25.3	7.91	992.09	1000.00	0.2
12	295	29.5	6.78	993.22	1000.00	0.2
13	379	37.9	5.28	994.72	1000.00	0.2
14	95.9	9.59	2.09	97.91	100.00	0.2
15	40	4	2.50	47.50	50.00	0.2
16	17.6	1.76	5.68	44.32	50.00	0.2
17	198	19.8	10.10	989.90	1000.00	0.2
18	52.1	5.21	3.84	96.16	100.00	0.2
19	137	13.7	14.60	985.40	1000.00	0.2
20	2.73	0.273	7.33	2.67	10.00	0.2
21	107	10.7	18.69	981.31	1000.00	0.2
22	125	12.5	16.00	984.00	1000.00	0.2
23	57	5.7	3.51	96.49	100.00	0.2
24	1.41	0.141	14.18	-4.18	10.00	0.2